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INCIDENCE OF LEUKEMIA IN PHYSICIANS

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INTRODUCTION

Early in the history of roentgen and gamma rays, it was noted that excessive exposure of the hands or other parts to high-energy radiation resulted in a dermatitis, which in some cases developed into carcinoma. In 1902 Frieben (*1*) reported the first case of radiation carcinoma, and in 1911 Hesse (*2*) was able to bring together reports on 94 such cases. Radiation injury to the blood and hemopoietic tissues was likewise noted at an early date. In 1904-5 Heineke (*3*), working with animals, observed degenerative changes in bone marrow both of the lymphoid and myeloid series. In a number of instances since that time, leukemia has been reported in human beings who have worked with high-energy radiation. Because of the similarity in character of the damage caused in hemopoietic tissues to that caused in epithelium and because epithelial damage in some cases resulted in malignancy, authors were quick to recognize the possible etiologic significance of radiation in leukemia.

In 1942 Dunlap (*4*) reported that 24 cases of leukemia in radiation workers were found in the literature:

> The 24 victims ranged in age from 29 to 53 years; 20 were men and 4 were women. All had had years of occupational exposure to radiation and several showed radium or roentgen burns of the hands. In 4 of the cases, leukemia followed exposure to radioactive substances and in the remaining 20 it was described as occurring in roentgenologists, radiologists and their assistants, who presumably had been working with X-rays. In 7 of the cases the condition was diagnosed as lymphatic and in 13 as myelogenous leukemia; in 4 the type was not specified. The disease in these patients did not differ in character and course from spontaneous leukemia. ***Only 3 autopsies are described, and many of the reports give such scanty details that the authenticity of the cases is open to question (*4, p. 577*).

Somewhat stronger evidence that radiation may bear a causal relationship to leukemia comes from experimental work with animals. Krebs, Rask-Nielsen, and Wagner (*5*) were the first to study this problem. They treated mice with X-rays and found the incidence of lymphomatosis among 5,500 irradiated animals to be 3.5 per thousand and that among nonirradiated controls to be 0.6 per thousand. Furth (*6*) gave whole-body treatments of 400 r single exposures at intervals of approximately 1 month and found an increase in leukemia (myeloid and lymphoid) of more than threefold. Hueper (*7*), using hard X-rays, gave general-body treatment to mice, doses up to 480 r being given in a 6-week interval, and observed an incidence of leukemia as high as 74 percent (in animals which he states had a high but unknown normal incidence of leukemia). Furth and Furth (*8*) gave single or repeated doses of 200 to 400 r of X-rays to the whole bodies of mice and observed an increase in myelosis of eightfold and in mediastinal lymphomatosis of sevenfold. The actual incidence of lymphomatosis and myelosis, however, did not exceed 13.7 and 7.6 percent, respectively. In work dealing with dosage in the induction of leukoses, Henshaw [1] found increases which in some cases exceed any thus far reported.

[1] Unpublished data.

There are, then, two types of evidence suggesting that high-energy radiation may increase the incidence of leukemia: (1) The fact that this disease has occurred in radiation workers under conditions resembling those in which radiation carcinoma has developed; and (2) the fact that the incidence of leukemia in animals has been increased experimentally by exposure to X-rays.

None of this evidence, however, furnishes any direct proof that radiation actually acts as a carcinogenic agent in the induction of leukemia in human beings. Although leukemia has been seen in persons having radiation employment, this fact alone cannot be taken as prima facie evidence that radiation incited the disease. It can be said only that it may have acted as the inciting agent. Since the leukemia found in irradiation workers appears to differ in no significant respect from that found in other persons and since the experimental investigation of this question is not possible in human beings, the problem is not open to direct study and necessitates the use of less direct methods.

As one approach, it would be desirable to compare statistically the incidence of leukemia in two groups of individuals, one occupationally exposed to significant amounts of radiation and the other not so exposed. The most suitable exposed group for such a study would be radiation engineers, miners of radium ore, radiation physicists and technicians, radiologists, etc. So far as we know, however, satisfactory health and death records are not available for significantly large groups of such persons.

Since true leukemia is eventually fatal, a study of deaths from leukemia gives a direct measure of incidence of the disease. Hence it would appear that further information might be obtained by utilizing the death notices of physicians, published each week in the Journal of the American Medical Association, and comparing the results obtained with similar material for the general population, taken from the vital statistics of the United States Bureau of the Census. Although many physicians never work with X-rays or radium and although the population as a whole includes all persons exposed to high-energy radiation as well as those who are not, it may be presumed that physicians as a group are more exposed to radiation than is the average for the total population. With these points in mind, we undertook to determine the incidence of leukemia in the two groups under conditions as nearly comparable as possible with the available sources of data.

EXPERIMENTAL PROCEDURE

A survey was made of the death lists given in the Journal of the American Medical Association for the 10-year period 1933-42, inclusive, and a record made of various items, as shown in table 1. The various types of leukemia as given in the journal are listed and taken together constitute the main object of interest in this study. The other diseases listed are of related interest, but since appropriate control data are not available, deaths attributed to them are being placed on record for possible future use only. The table also gives the number of deaths that were published; the number of deaths for which causes were given; and the number of deaths from cancer (all types) and also the percentage of deaths.

Because death rates vary with age and because age distributions of the test and control groups may differ in certain respects, it was necessary to obtain age distributions in order that comparisons could be made on an age basis. In table 2, the usable data of table 1 have been arranged according to 5-year age groups. Table 2 gives the number of deaths from all causes, the deaths from cancer, the deaths from leukemia, the ratio (in percentage) of deaths from leukemia and deaths from cancer (all types), and the ratio (in percentage) of deaths from leukemia to total deaths.

It was necessary to select from the material for the general population the kind of data most suitable for comparison, as the Bureau of the Census gives separate listings by sex and color as well as by age. Although the incidence of leukemia is higher in female and in colored groups, and although the physicians'

TABLE 1.—*Deaths of physicians* [1]

Cause of death	Number of deaths during the years—										
	1933	1934	1935	1936	1937	1938	1939	1940	1941	1942	Total
Leukemia	4	5	10	4	5	10	7	8	3	1	57
Lymphatic leukemia	1	1	6	3	6	4	7	3	3	3	37
Myelogenous leukemia	5	3	2	3	2	5	4	9	5	3	41
Monocytic leukemia	0	0	0	2	0	2	1	1	2	0	8
Total	10	9	18	12	13	21	19	21	13	7	143
Lymphosarcoma	1	3	4	2	2	1	7	3	5	4	32
Lymphoblastoma	1	0	0	0	0	1	0	1	1	0	4
Myeloma	2	2	0	0	0	0	3	0	4	2	13
Hodgkin's disease	5	7	4	4	4	5	8	1	2	4	44
Aplastic anemia	0	2	1	1	1	7	5	1	4	1	23
Pernicious anemia	11	11	5	6	5	12	10	9	9	3	81
X-ray burn	2	1	3	3	1	0	1	0	1	1	13
Total deaths—number	3,355	3,392	3,492	3,583	3,393	3,748	3,882	3,640	3,482	3,331	35,298
Deaths (causes given) number	2,740	2,683	2,722	2,822	2,571	2,967	2,917	2,637	2,488	2,241	26,788
Deaths from cancer {number [2]	227	220	228	224	222	282	282	264	273	221	2,443
Deaths from cancer {percent [3]	8.3	8.2	8.4	7.9	8.6	9.5	9.7	10.0	11.0	9.9	9.1

[1] Compiled from death notices in the Journal of the American Medical Association, 1933–42, inclusive.
[2] All types, including the leukemias, lymphosarcoma, lymphoblastoma, and myeloma, but excluding Hodgkin's disease.
[3] Based on deaths for which cause was given.

group includes females and colored persons, material for white males was selected as most suitable, and no corrections were made for the female and colored components.

The Bureau has from time to time changed its methods of classifying and reporting data. Because of such changes in 1939, the data for 1939 and 1940 were selected as most satisfactory for our purposes, inasmuch as this 2-year period was near the middle of the interval in which we were interested and the number of deaths was large. The figures are shown in table 3, the same plan of presentation being used as in table 2. The values for deaths from cancer (all types) pertain to the total of the two groups listed in the mortality reports under the headings Cancer and Other Malignant Tumors (rubrics 45-55) and Leukemia (rubric 74).

RESULTS

The totals in table 2 show that 26,788 physicians, for whom the cause of death was given, died during the period studied; 2,443 of these deaths were due to cancer, and of these 143 were due to leukemia. Thus, 0.53 percent of the total deaths and 5.9 percent of the deaths from cancer were ascribable to leukemia. Table 3 shows that 1,361,786 white males of the general population, for whom the cause of death was given, died during 1939-40; 144,840 of the deaths were due to cancer, and 5,286 to leukemia. In this case, 0.39 percent of the total deaths and 3.6 percent of the deaths from cancer were attributed to leukemia.

TABLE 2.—*Deaths of physicians* [1]

Age range (in years)	Deaths with cause given	Deaths from cancer (all types) [2]	Deaths from leukemia [3]	Ratio of deaths from leukemia to deaths from cancer	Ratio of deaths from leukemia to deaths with cause given
	Number	*Number*	*Number*	*Percent*	*Percent*
20–24	19	1	0	0	0
25–29	257	11	4	36.4	1.56
30–34	449	30	12	40.0	2.67
35–39	551	32	8	25.0	1.45
40–44	866	48	8	16.7	.92
45–49	1,243	93	9	9.7	.72
50–54	1,948	135	13	9.6	.67
55–59	3,049	279	15	5.4	.49
60–64	3,963	395	25	6.3	.63
65–69	4,207	433	22	5.1	.52
70–74	3,720	400	12	3.0	.32
75–79	3,061	321	9	2.8	.29
80–84	2,102	191	4	2.1	.19
85–89	974	60	1	1.7	.10
90–94	323	14	1	7.1	.31
95–99	44	0	0	0	0
100–	12	0	0	0	0
Total or percent	26,788	2,443	143	[4] 5.9	[4] .53

[1] Compiled from the deaths listed in the Journal of the American Medical Association for the period 1933–42, inclusive.
[2] Including the leukemias, lymphosarcoma, lymphoblastoma, and myeloma, but excluding Hodgkin's disease.
[3] Leukemia and lymphatic, myelogenous, and monocytic leukemia.
[4] Based on totals.

TABLE 3.—*Deaths in the general population*[1]

Age range (in years)	Deaths with cause given	Deaths from cancer (all types)[2]	Deaths from leukemia[3]	Ratio of deaths from leukemia to deaths from cancer	Ratio of deaths from leukemia to deaths with cause given
	Number	*Number*	*Number*	*Percent*	*Percent*
0–4	123,892	975	491	50.4	0.396
5–9	11,488	607	273	45.0	2.38
10–14	11,752	514	202	39.3	1.72
15–19	19,059	789	228	28.9	1.197
20–24	23,559	899	160	17.8	.679
25–29	24,835	1,084	165	15.2	.664
30–34	28,494	1,541	179	11.6	.628
35–39	35,611	2,396	225	9.4	.631
40–44	48,946	4,445	274	6.2	.560
45–49	70,330	7,748	370	4.8	.526
50–54	94,657	12,252	443	3.6	.468
55–59	114,450	16,201	453	2.8	.396
60–64	133,224	20,023	558	2.8	.419
65–69	152,526	22,466	472	2.1	.310
70–74	155,631	21,632	371	1.7	.238
75–79	140,306	17,230	267	1.5	.191
80–84	103,997	9,657	127	1.3	.122
85–89	50,391	3,513	21	.6	.042
90–94	15,331	739	7	.9	.045
95–99	2,896	129	0	0	0
100–	411	0	0	0	0
Total or percent	1,361,786	144,840	5,286	[4] 3.6	[4] .39

[1] Compiled from U. S. Bureau of the Census reports for 1939–40.
[2] Including the leukemias, lymphosarcoma, lymphoblastoma, myeloma, but excluding Hodgkin's disease.
[3] Leukemia and lymphatic, myelogenous, and monocytic leukemia.
[4] Based on totals.

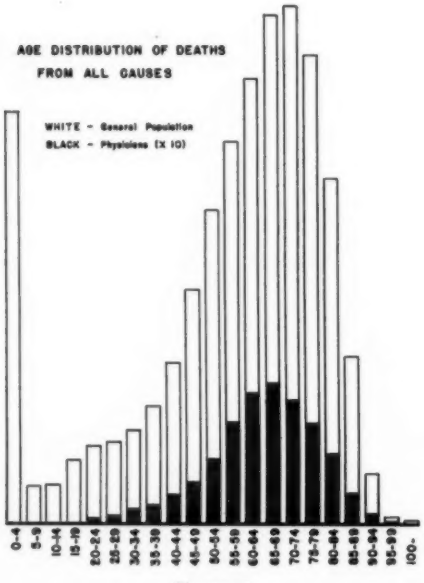

FIGURE 1.

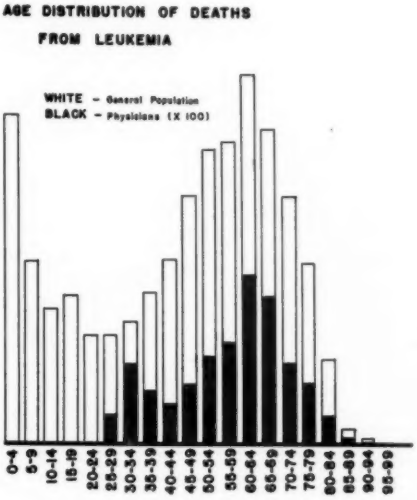

FIGURE 2.

While these findings provide one type of comparison, it was apparent that more reliable comparisons could be made by analyzing the data on an age basis. Figure 1 shows the age distribution of deaths from all causes, the data being taken from tables 2 and 3. The values for physicians have been increased by a factor of 10 for the graph in order that the distribution can be seen more plainly.

The picture for the general population is the familiar one, the number of deaths being high in infancy, relatively low during childhood, but increasing again with age to a peak at 65 to 75 years of age. As would be expected, there were no deaths among physicians before the age of 20. Beyond this age, the distribution of deaths was generally similar to that for the general population except that the greater number of deaths occurred at a slightly earlier age, 60 to 70 rather than 65 to 75.

Figure 2 shows the age distribution of deaths from leukemia. In this case the values for physicians were increased by a factor of 100 to facilitate visual comparison. This graph shows that the number of deaths from leukemia (for the general population) was high in early childhood, that it dropped to a low at the ages of 20 to 30, and that it rose again,

reaching a peak at 60 to 65. Above the age of 40, the material for physicians showed essentially the same pattern.

In the last two columns of tables 2 and 3, the ratios of deaths from leukemia to deaths from cancer, and of deaths from leukemia to deaths from all causes are given for the different age groups. The values obtained for ages up to and including 84 were plotted in figures 3 and 4,

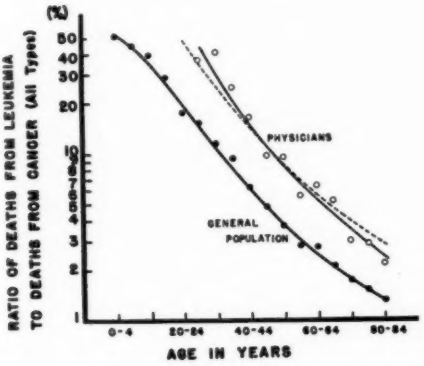

FIGURE 3.

respectively. Beyond the age of 84 the data were too unstable to be of interest.

In figure 3 the curves for the general population show that approximately 50 percent of all cancer deaths in persons under 5 years of age were due to leukemia and that as age increases, the ratio of leukemia to total deaths decreases. The curve for physicians follows the same general course but lies distinctly above that for the general population, indicating a higher incidence of leukemia. The solid line shows the best smooth curve that could be drawn among the points by inspection, and the dotted line is drawn with a constant relationship to the curve for the general population. The fact that the points in the two cases follow so nearly a parallel course when plotted on a semilogarithmic scale shows that the factor of difference for the two groups was nearly constant for all ages.

The extent of difference may be obtained by ascertaining the ratio at some representative age. By taking this at 60, where the curves are most stable, the incidence of leukemia in physicians was found to be 2.2 times greater than in white males of the general population.

In figure 4, deaths from leukemia are compared with total deaths. In the general population, the ratio of deaths from leukemia to deaths from all causes is relatively low during infancy, high during childhood, and drops off rapidly during adolescence. The lowness of the point for 0-4 years might appear at first to be at variance with the results in figure 3. However, it should be understood that its location is determined largely by high infant mortality from causes other than cancer. Beyond the age of 25, the ratio of deaths from leukemia to deaths from all causes decreases with age. Again, all the points for physicians fall distinctly above the curve for the general population. Although the first three points for the physicians' data appear to be proportionately higher than the other points, it must be remembered that the data for the youngest as well as the

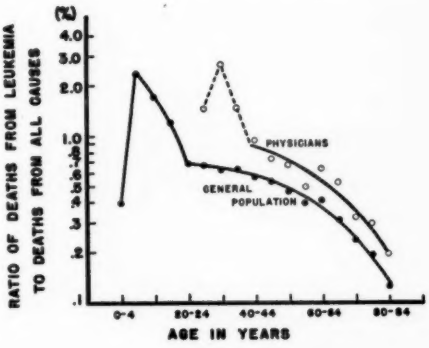

FIGURE 4.

oldest age groups are less stable than those for the intermediate. For the age period 40 to 84 years the physicians' curve was drawn with a constant relationship to the curve for the general population. Since this curve fits the points as well as any that could be drawn, the relative incidence of leukemia in the two groups can be determined by taking the ratio at any point along the

curves. It was found in this case to be 1.5 times higher in physicians.

Thus, from two types of comparison, the incidence of leukemia in physicians was found to be higher than in the general population (2.2 and 1.5 times higher, respectively, by the first and second methods). Since, however, the differences observed were entirely relative, it was impossible to say whether they were due actually to more leukemia or to less deaths from other causes in physicians. From tables 2 and 3 (age groups 40 to 84, inclusive) it may be determined that only 9.5 percent of the physicians died of cancer while 13.0 percent of the general population died of this malady, indicating that the difference in part at least was due to less cancer in physicians.

In order to test the data still further, a third method of analysis was used. In this case, the death ratios were calculated. Figures for the total population were obtained from the Census Reports for the year 1940, and figures for leukemia (table 3) were adjusted to give the average number in 1 year. The death ratios from leukemia in the total population were then calculated for the 5-year age groups from 25 to 74 years, all above 74 years being grouped. The values were plotted in figure 5 as solid circles. Similar figures for the number of physicians living during the year 1940 were obtained from unpublished compilations of the Division of Public Health Methods, United States Public Health Service, and related to the figures for leukemia in physicians (table 2), the latter being divided by 10 to give the average yearly incidence. Death rates based on these values are plotted as open circles in figure 5.

Again the points for physicians fall above those for the general population. The best smooth curve was drawn by inspection through the points for the latter. From further inspection, it became apparent that the best curve through the points for the data for physicians would run nearly parallel to that for the general population and also that the ratio of difference in incidence would be very nearly 1.5, the same as that obtained by comparison on the basis of deaths from all

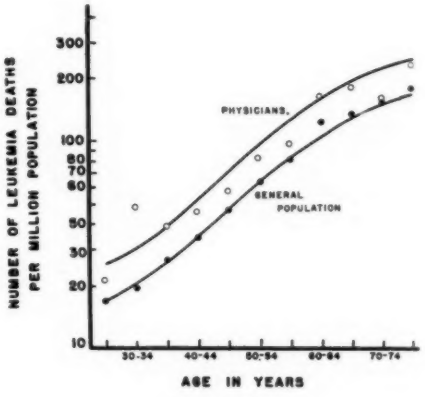

FIGURE 5.

causes. The upper curve, therefore, was drawn to show a constant difference of 1.5. When the curve is dropped so that an equal number of points in this middle section appears on either side, the ratio becomes 1.3; but this value needs further correction.

Table 1 shows that while 35,298 physicians died during the period 1933-42, the cause of death was reported for only 26,788, or 76 percent. Thus, since the control data obtained from the Public Health Service pertained to all physicians living in the period considered, it was clear that all the points for the data for physicians (fig. 5) should be raised by a ratio of 76 : 100. When such adjustments were made, the value 1.3 was changed to 1.71. This figure may be taken as the best estimate made on the basis of death rates; and since it is intermediate to those obtained previously, it probably represents the most satisfactory figure indicating the relative incidence of leukemia in physicians that can be obtained at this time.

DISCUSSION

It is recognized at the outset that the methods of obtaining and reporting data used by the American Medical Association and the United States Bureau of the Census are not identical. Since physicians' deaths are reported mainly as news items, the deaths are sometimes announced before a statement as to the cause of death is at hand. As already

indicated, the cause of death was given in only 26,788 of the 35,298 cases reported, a difference of 8,510. If we assume that leukemia might be more promptly reported than other causes (causes which might reflect on the integrity of the individual) and if we go so far as to assume that none of the 8,510 doctors had leukemia, on the basis of the age distributions at our disposal, the differences observed would be reduced appreciably although in no case would they disappear completely.

This matter was taken up with the American Medical Association, and we were told (1) that the association exercises no selection whatsoever in reporting causes of death, and (2) that the records concerning causes eventually become very nearly complete. In order to answer the question whether selection of some kind was inadvertently exercised by friends or relatives reporting the deaths, the association very kindly went through its more complete files for the year 1942 and obtained figures for leukemia. The total figure was 18 (leukemia, 4; myelogenous leukemia, 6; lymphatic leukemia, 8; monocytic leukemia, 0), whereas our total was 7 (table 1). It should not be presumed that so great a difference would be maintained for the other years if complete data were available, as table 1 shows that our value of 7 is unusually low for 1942. It seems unlikely, therefore, that any selection was used either inadvertently or otherwise, and that the differences observed would not be significantly reduced if complete information on physicians were available.

Very few published data are available pertaining to the incidence of leukemia among physicians. One report (Decennial Supplement, Part IIa, Occupational Mortality, Report of the Registrar General of England and Wales, 1931) shows that five deaths from leukemia occurred among English and Welsh physicians, ages 20-65, where three would have been expected on the basis of ages of all males in those years. Of related interest was the fact that males in the professional classes as a whole had an incidence of leukemia 53 percent above the average, whereas the unskilled workers had an incidence 15 percent below the average. In a comment on these observed differences it is stated:

> Differing precision of diagnoses of these diseases in the different social strata might account for some part of these variations, but it seems unlikely that it could account for the great contrasts which are observed.

Whether or not differences in these incidence rates of leukemia are in any way attributable to radiation is unknown, but the results of this English study make it desirable that data pertaining to physicians be controlled by data on professional groups not potentially exposed to radiation. This could not be done with the material at our disposal.

If we accept the available evidence that leukemia is higher among physicians than among the general population, it does not follow necessarily that radiation acted as the inciting agent. Eventually it may be found that some factor quite apart from radiation is responsible for the apparent higher incidence. It has been suggested that if radiation were responsible for the greater proportion of leukemia in physicians, the continued exposure during their lifetimes should produce a continually increasing difference with age, making the curves (figs. 3, 4, and 5) divergent. But until more is known about the mechanism of radiation-induced neoplasia (the significance of dosage, rate of administration, methods of exposure, biologic factors, etc.), it will be impossible to discuss this feature with profit.

Perhaps the most significant aspect of this study is the fact that the findings are in accord with experiments with animals. Animals receiving single or repeated doses of a few hundred r showed a higher incidence of leukemia. Judging from the series of recent reports dealing with the problem of stray radiation around X-ray and radium units (*9-13*), we find that doses in the same range are frequently received by operators in a period of days, months, or years. The full meaning of the data presented can

become apparent only after further developments.

SUMMARY

A comparison was made of the incidence of leukemia in physicians and in the general population.

Data were obtained from the death lists of the Journal of the American Medical Association, from the mortality reports of the United States Bureau of the Census, and an unpublished compilation of the United States Public Health Service.

Comparisons were made on the basis of (1) the ratio of deaths from leukemia to deaths from cancer, (2) ratio of deaths from leukemia to total death rates, and (3) death rates from leukemia.

Leukemia was recognized approximately 1.7 times more frequently among physicians than among white males in the general population.

Possible discrepancies in the data were discussed.

While these observations furnish no direct proof that radiation acts to incite leukemia in human beings, they are, nevertheless, in accord with the findings on experimental animals in which exposure to X-rays has been found to increase the incidence of leukemia.

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ORGANIZATIONAL STRUCTURE AND ACTIVITIES OF STATE CANCER PROGRAMS [1]

By JAMES W. HAWKINS, *passed assistant surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

The National Cancer Institute, since 1938, has closely observed the extension of cancer-control programs conducted by State health agencies. The purpose and scope of these activities were reviewed in 1941.[2] Considerable thought and attention by outstanding medical and public health leaders have entered into this development. Thousands of cancer patients have been assisted. Although the results of these control programs cannot as yet be fully evaluated, tentative generalizations concerning some aspects can now be made.

On the basis of information supplied by State health departments and State cancer commissions, the State activities are outlined herein according to the legislative backgrounds of each and apply to the fiscal year 1942-43. In view of the fact that a summary has recently been completed of all State legislation pertaining to cancer control and is available in mimeographed form through the National Cancer Institute, no further reference to the nature or text of such legislation will be made here. State cancer-control activities are classified according to the presence of cancer-control laws, of personnel working specifically on cancer, and the type of administrative organization. No attempt has been made to allocate for budgetary purposes that proportion of time spent by full-time health-department personnel on cancer activities, except when such activities were their principal or only function.

STATES WITHOUT CANCER-CONTROL LAWS

States having no cancer-control laws and no personnel working specifically on cancer are here considered according to the control activities conducted as a part of their broad public health program. Those that do not have cancer programs are: California, District of Columbia, Kentucky, Maryland, Nevada, North Carolina, Pennsylvania, South Dakota, Utah, Virginia, and Wyoming. In nine States the only activity is an educational program. These are: Arizona, Idaho, Indiana, Minnesota, Nebraska, North Dakota, Ohio, Tennessee, and Washington. In one State, New Mexico, cancer reporting is the only activity. In another State, Arkansas, there are both cancer reporting and tissue diagnostic service. Six States, Delaware, Wisconsin, Florida, Kansas, Montana, and Mississippi, conduct both cancer reporting and an educational program; Mississippi also has a part-time clinic. Louisiana requires cancer reporting and conducts an educational program and a tissue diagnostic service.

Among these 29 States, only 2, Idaho and Montana, carried appropriations for cancer, $100 and $200, respectively, for cancer education. While in some States the designated activities were rather extensive, in others the program was in name only.

Six States not having cancer-control laws have had one or more full- or part-time personnel working on cancer:

Oklahoma conducted a lay and professional educational program, supported one small clinic, and follow-up service was furnished by one medical social worker. This program was administered by an epidemiologist, whose activities were discontinued December 31, 1942. The total budget for this program for one-half year was $2,006.

New Jersey's full-time trained officer left the State for duty with the armed forces. The budget for this activity for one-half year, $2,100, was discontinued December 31, 1942.

Michigan has a full-time officer in cancer control and conducts a program of lay and professional education. The expenditure was $6,928.58.

The program in Oregon, conducted by a part-time medical director and three full-time assistants, consists of education, three clinics with follow-up service, and statistical research. The budget was $5,215. Laboratory research has been discontinued in Oregon.

In Colorado there are two full-time employees. The program covers education, reporting, diagnosis, and treatment and is con-

[1] Presented at the cancer symposium of the American Public Health Convention in New York City, October 11, 1943.

[2] SCHEELE, L. A.: Present status of state cancer control programs. Pub. Health Rep., 57: 1613–1620 (1942).

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ducted for the most part by the Women's Field Army of the American Society for the Control of Cancer. The budget for these activities was $6,235.

In Iowa the medical officer in charge of the cancer-control activities has recently been given administrative responsibilities aside from cancer control. However, the programs of lay and professional education, tissue diagnostic service, and support of diagnosis and treatment clinics have been continued. The number of clinics has been reduced from five to four. The budget was $31,610.00, of which over $17,000 was from local funds.

In these six States, the total budget included local, State, and Federal funds authorized by Title VI of the Social Security Act. Over half of the total budget was from the State of Iowa.

STATES WITH CANCER-CONTROL LAWS

States with cancer-control laws have programs administered either by State cancer commissions or by divisions of State health departments.

In all three instances, the cancer commission is an administrative division of State government, the only activity being cancer control:

The program in Missouri has been conducted by a full-time secretary, whose primary responsibility has been the administration of a State cancer hospital. Diagnosis, treatment, and follow-up services are the principal activities, and an educational program is also carried on. The total budget was approximately $224,000.

In New Hampshire, the program is headed by a part-time physician, and there are two full-time employees. Support is given to tissue diagnostic service through private laboratories and 14 clinics, of which 7 give therapy, limited follow-up service, and terminal care given either in the patient's homes, or in nursing homes. The budget was $42,014.88. The reporting of cancer morbidity has been discontinued.

The program in Vermont is headed by a part-time physician and three full-time employees and consists of lay and professional education, tissue diagnostic service in central and private laboratories, three clinics, and follow-up service. The budget was $20,000.

These three States with programs administered by cancer commissions have emphasized the diagnostic and treatment phases of cancer control.

In 10 States, the cancer laws designate that control activities be conducted by the State health department. Seven States, Connecticut, Georgia, Illinois, Maine, Massachusetts, New York, and South Carolina, have programs; and in 3, Alabama, Rhode Island, and West Virginia, cancer legislation has been so recent that the programs are not as yet developed.

In Connecticut, there is a full-time officer in charge, with four full-time and three part-time employees. The program includes lay and professional education, a tissue diagnostic service given through private laboratories, support of 23 diagnostic and treatment clinics, follow-up service, and statistical research. The expenditure was $42,442.93.

In Georgia, the staff consists of a full-time officer and two full-time assistants. The program consists of education, chiefly for the lay public, tissue diagnostic service available through private laboratories, 12 diagnostic and treatment clinics, hospitalization for indigents, follow-up service, and statistical research. The expenditure was $95,242.56.

In Illinois, the program is headed by a full-time medical officer who since March 1942 has been given additional duties in the health department. He has 3 full-time and 16 part-time employees in clinics and conducts a lay and professional educational program, tissue diagnosis in both central and private laboratories (the central laboratory being recently discontinued), 4 diagnostic clinics with therapy available elsewhere, follow-up service, and a tumor registry. The expenditures totaled $23,064.49.

In Maine cancer control is one of the activities conducted by the division of medical service, which supplies medical social workers for cancer and conducts lay and professional education, tissue diagnosis by private laboratories, aid to cancer clinics, follow-up service, and statistical research. Only $3,381 of the division's budget was specifically for cancer.

In Massachusetts cancer control is the principal activity of the division of adult hygiene, and is administered by a full-time officer, 25 full-time employees, and 1 part-time. There are an extensive program of professional and lay education, tissue diagnosis through a central laboratory, 21 diagnostic clinics of which 2 also give treatment, a special cancer hospital and additional facilities in another hospital, and statistical research. The budget was $516,655.81.

The program in New York is conducted by a full-time officer and 16 full-time employees, excluding hospital personnel. The reporting of cancer cases is required by law. The rather extensive program consists of lay and professional education, central laboratory diagnostic service, 37 clinics, a special cancer hospital, follow-up service, and both statistical and other research. Expenditures were $538,614.29.

In South Carolina the program is administered by a full-time officer, who now has responsibilities aside from cancer control, and nine part-time employees. The activities are professional education, tissue diagnostic service given through private laboratories, diagnos-

tic clinics, hospitalization for indigent patients, follow-up service, and statistical research. The budget was $33,908.37.

Cancer control laws have been enacted so recently in Alabama, Rhode Island, West Virginia, and Texas, that no attempt is made to summarize the current activities of these States. In Texas, the law designates that the funds appropriated for a State cancer hospital and division of cancer research be administered under the supervision of the University of Texas; other aspects of cancer control are conducted by the State health department. The budget in these four States totals $472,500. A shortage of available physicians trained in cancer control is contributing to considerable delay in establishing these programs recently authorized.

CONTROL ACTIVITIES

Recognition of the importance of lay and professional education is emphasized by the fact that this activity is extensively carried on in most States without regard to the presence of State cancer-control legislation. In some States the educational programs are conducted practically entirely by the official State agency, in others principally by the Women's Field Army of the American Society for the Control of Canger, but in most States this activity is cooperative. State and local medical societies are playing an important role not only in the educational aspects of cancer but also in all of the associated service activities and have done much to promote and sponsor the existing control programs.

The reporting of cancer cases is required by regulation of the State health departments in New Mexico, Arkansas, Florida, Kansas, Montana, Colorado, Mississippi, and Louisiana and is required by law in Delaware, Wisconsin, New York, and Rhode Island. Only the 2 last-mentioned States have cancer-control legislation covering broad control programs. Among these 12 States, 1, New York, presents evidence of relatively complete reporting, and in New York a rather reliable index of cancer prevalence is now being obtained. Connecticut does not require that cancer be reported but is obtaining rather complete morbidity records by a direct, continuous survey of the various hospitals in the State that accept cancer patients. For the purpose of planning control programs, sufficiently accurate estimates of cancer prevalence can now be made on the basis of mortality data, utilizing for interpolation the experience in these 2 States and the recent surveys of various metropolitan areas by the United States Public Health Service.[3] Cancer reporting is not a service activity, and up to the present has had limited value except when completely practiced, when associated with broad control programs, or in so far as it has stimulated the development of more direct cancer service.

With few exceptions, direct service to patients with cancer is seldom adequately supported by State health agencies in States not having specific cancer-control legislation. Such service involves tissue diagnosis, the organization and subsidy of diagnostic and treatment clinics, the hospitalization of cancer patients and follow-up, and requires trained professional administration and adequate appropriations.

Tissue diagnostic service is being made available to physicians and hospitals in several ways. In some States this service is given through the laboratory division of the health department, in others through private pathologists and pathologic laboratories on a part-time salary or fee basis, and in others through subsidy of this service in hospitals having approved cancer clinics.

The concept of approving clinics in general hospitals having adequate organization, personnel, and equipment for the diagnosis and treatment of cancer has been developed by the American College of Surgeons. The acceptance of these principles by hospital staffs has done much to promote improved cancer care. On the other hand, the recognition of these ideals is but the first step in the diagnostic and treatment program. The various elements of the program require financial support, frequently beyond that which can be made available by indi-

[3] Dorn, H. F.: Pub. Health Rep., 59: 33.

vidual hospitals or by subscription. The State cancer-control organizations not only are recommending the establishment of approved clinics but are aiding and supplementing the existing facilities where needed so that the necessities incident to more modern cancer care are being made available. Most of the cancer patients who are aided directly or indirectly by the State programs actually receive diagnostic and therapeutic services in these approved clinics and hospitals which are neither owned nor operated by the State.

Three States, Massachusetts, Missouri, and New York, conduct State cancer hospitals, and a fourth, Texas, has made provisions for one.

Although the clerk of the cancer clinic can keep in touch with most of the cancer patients, complete follow-up is seldom attainable without the services of a medical social worker. In States where the program is administered in the health department, the public health nurses are available for home visits.

One State, New York, conducts laboratory research; provisions for such research have recently been made by Texas. While laboratory research is fundamental to the development of our basic knowledge of cancer, such research is not usually considered to be an essential component of State control programs, but rather a function of large educational institutions, endowed organizations, and federally supported agencies such as the National Cancer Institute. On the other hand, statistical research relating to the evaluation of control activities and to epidemiologic aspects of cancer is an important phase of many of the more complete State cancer-control programs.

The administration of broad control programs requires training and experience both in cancer and in public health procedures. With this in mind, the Harvard School of Public Health has developed a course in cancer control which involves field experience in the Massachusetts program. Termination of the war will not give immediate relief to the existing personnel problems. It is evident that States are finding it necessary to assume the responsibility for supplementing the training of physicians with good medical backgrounds so that adequate leadership will be available as needed.

SUMMARY

In 38 States cancer-control programs are conducted by official agencies of the State government. Educational activities predominate.

In the last 2 years, the budget for cancer has doubled; the budget for the fiscal year 1942-43 totaled $2,066,900.33, of which 97 percent was in the 14 States having cancer legislation.

The development of leadership for cancer programs and the promotion of appropriate control legislation appear to be the most important steps in the establishment of adequate State cancer-control programs.

MEASUREMENT OF EPITHELIAL GROWTH IN SURGICAL WOUNDS OF THE RABBIT'S EAR

By PAUL S. HENSHAW, *senior radiobiologist*, and HENRY L. MEYER, *scientific aide, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The rate of closure of epithelial wounds has been a subject of study by numerous investigators (*1-20*). In these instances, with one notable exception (Lumiére), the amount of coverage of a wound, that is, the amount of area healed, has been studied as a function of time.

The area approach to the problem of measuring healing rates was initiated and developed largely by Carrel and Du Noüy (*1, 4, 5, 13, 17*), who pointed out that the increment of area healed per unit time is higher at the beginning of a healing process than at the end, and that the rate of repair (in terms of area) is greater in large wounds than in small ones. Lumiére (*8, 10, 11*), on the other hand, measured epithelial wounds in terms of breadth and pointed out that the healing rate is essentially constant during the main healing period.

During the course of some work with ointments, we found it necessary to give attention to the healing process and to the different methods of measuring wound growth. This work proved to be of particular interest in itself, as it necessitated consideration of certain basic aspects of wound behavior. This report presents results of the studies carried out, giving at the same time the comparisons of different methods of measurements. Since the first draft of this paper was written, a report by Howes (*22*) has been published dealing also with methods of measuring epithelial growth in wounds. Because of the close similarity between certain of Howes' work and some of our own, this report is being condensed somewhat and reference made to Howes' results when the two studies run parallel.

GEOMETRICAL CONSIDERATIONS

It is known that epithelial growth in a wound takes place at the wound borders and that the granulation tissue bed, if free of epithelial elements, contributes in no way to epithelial coverage as such. Likewise it is known that the epithelial edges move laterally from the original wound borders to cover the exposed granulation tissue. Geometrically, then, two basic features become apparent: (1) The wound perimeter becomes shorter as the coverage proceeds; and (2) coverage becomes complete when the epithelial sheets meet at middle points.

Since epithelial growth takes place at the borders and since the epithelial sheets move by lateral extension, a question arises which bears directly on the problem of coverage rates. Consider the rate factor as illustrated by figure 1, *A* and *B*. If epithelial growth is straight

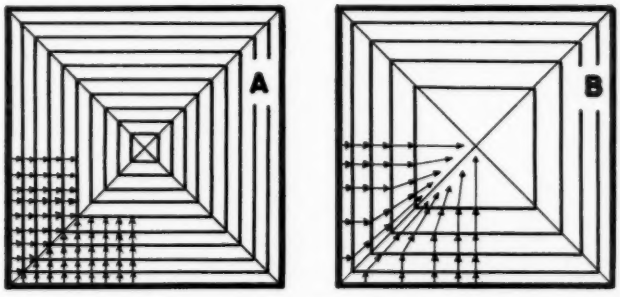

FIGURE 1.—Two possible types of epithelial growth in square wound areas. *A*, Coverage as it would be if lateral extension took place at a uniform rate and growth activity ceased when growth lines intersect. *B*, Coverage as it would be if the same amount of area were covered for equal increments of time (the area between each square is equal to that of the central square).

out from the borders, what happens when the growth lines intersect as in the corners of wounds? Do the cells involved cease growth activity (fig. 1, *A*) or continue at an unabated rate (fig. 1, *B*)? It is obvious that if all the "healing forces" mobilized along wound borders at the beginning of wound repair continue to function at a fixed rate, the rate of lateral extension would increase as the perimeter becomes shorter. It is also obvious that if healing forces are immobilized when growth lines intersect, the rate of lateral extension would not necessarily be affected by a continually shortening perimeter. From these considerations, it is clear that the measurement of epithelial coverage rates, if done correctly, should yield important information regarding fundamental growth behavior in wound repair. As will be shown, however, the methods most commonly used fail to yield some of the most interesting material.

MATERIAL AND METHODS

Epithelial squares were removed surgically from the dorsal surface of the rabbit's ear following the procedure of Nettleship (*23*). By careful dissection, areas of skin can be removed in such a manner as to leave connective tissue and blood vessels essentially intact. Since a good connective tissue bed is present from the start in such wounds, inasmuch as infections are rare if the wounds are left exposed to the air, and very little cellular injury is involved, healing consists almost exclusively of epithelial regrowth. About 50 such wounds have been studied, but only representative groups will be considered.

RESULTS

EXPERIMENT 1

For this experiment, six rabbits 1 to 2 years of age were used. Areas approximately 20 x 20 mm. were removed from each ear. Immediately after the wounds were made and daily thereafter (except the first), measurements of the length and breadth were made with calipers for 14 days. In addition, daily photographs [1] were made (beginning on the third day), and daily notes were kept concerning each wound. When the experiments were complete, the photographs were assembled in a chart to show the daily progress of growth for each wound. Breadth values were available from the caliper measurements, and likewise area values were obtainable by determining the product of the length and breadth in each case. In addition area values were obtainable from the photographs by making planimeter readings on the wound surfaces shown. Thus, not only were the wound measurements made by two methods (breadths and areas), but the area measurements were obtained by two methods.

In work of this kind numerous factors render a wound unsatisfactory for study. At least four conditions may be listed as criteria for disqualification: (1) Secondary trauma; (2) infection; (3) epithelial upgrowth from the floor of the wound (due to incomplete removal of epithelium); and (4) exposure of ear cartilage (caused by cutting too deeply into the connective tissue). Six of the twelve wounds of this series were eliminated for one or the other of these reasons, leaving six others the data for which will be analyzed.

TABLE 1.—*Linear measurements and calculated areas for epithelial wounds of the rabbit's ear*

Growth (in days)	Wound No.— 6L	7R	8L	8R	9R	11R	Average area
	Millimeters	*Millimeters*	*Millimeters*	*Millimeters*	*Millimeters*	*Millimeters*	*Square millimeters*
0	21x21	22x20	23x20	22x20	21x18	21x21	433
2	21x21	21x19	23x21	20x19	22x20	21x19	424
3	21x20	21x19	23x21	20x19	21x20	21x19	417
4	20x18	21x19	22x20	20x18	19x18	20x20	384
5	18x17	20x17	19x16	18x15	15x14	17x17	287
6	15x15	19x16	18x16	15x14	14x12	19x17	255
7	12x12	20x16	15x13	11x10	9x8	14x14	171
8	12x10	18x13	11x11	8x8	7x7	13x11	119
9	9x9	16x11	9x8	8x7	7x7	12x7	86
10	7x7	15x10	9x6	6x6	4x4	10x6	61
11	6x5	13x8	7x5	5x4	5x4	7x3	38
12	4x4	14x8	6x4	4x3	4x4	5x3	33
13	5x4	13x7	5x4	4x3	4x3	4x3	28
14	4x3	11x6	5x4	3x2	4x2	3x2	20

Table 1 gives measurements of the lengths and breadths of the six wounds used together with average areas.

[1] Since the photographic method used was not greatly unlike that used by Howes, it is not being described here.

Planimeter measurements were made of the wound areas shown in the photographic chart. The values obtained, together with averages, are shown in table 2. In order to change the planimeter readings (for the averages) into square millimeters and at the same time adjust for photographic enlargements, a correction factor was necessary. This factor was given the value 4.16; the first planimeter value (i. e., for the third day) then equaled that obtained by means of the linear measurements (on the third day). By making this same correction for all the planimeter values, a set of figures was obtained as shown in the last column of table 2. These represent measurements in square millimeters and are comparable with those obtained by calculations from the linear measurements. Thus in tables 1 and 2 we have breadth measurements, and area measurements obtained by two methods.

TABLE 2.—*Planimeter readings from photographs*

Growth (in days)	Wound No.—							x4.16 [1]
	6L	7R	8L	8R	9R	11R	Average	
								Square millimeters
3	103	96	112	88	101	99	100	416
4	90	94	106	93	81	96	93	389
5	58	69	74	55	54	77	65	270
6	50	76	72	49	48	76	62	258
7	33	64	40	26	19	44	38	158
8	22	52	38	15	10	32	28	116
9	17	42	20	13	12	12	19	79
10	12	36	16	9	6	9	15	62
11	7	27	8	5	5	8	10	42
12	4	24	4	4	2	2	7	29
13	3	21	2	2	2	1	5	21
14	2	10	1	1	1	1	3	12

[1] Correction factor covering both the photographic enlargement and the planimeter units.

The averaged values of the area data (table 1) were plotted in figure 2 as solid circles. In similar manner, the averaged and corrected values (table 2) were plotted in the same figure as open circles. As seen in figure 1, the two sets of points are similarly distributed, and all points fit closely to a smooth curve drawn among them. This means that the values obtained by the less cumbersome caliper method were as consistent and satisfactory as those obtained by the photographic and planimeter method.

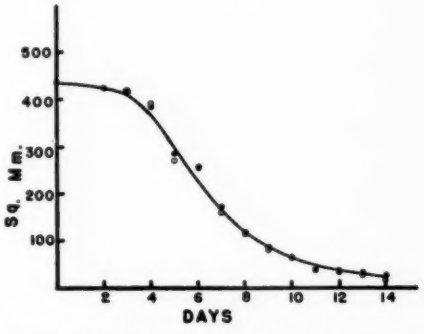

FIGURE 2.—Curve showing how the area of epithelial wounds on the rabbit's ear varies with time. The solid circles represent values obtained as the product of length and breadth measurements, and the open circles values obtained by making planimeter measurements on the photographs (with corrections).

The curve as drawn has a short plateau at the beginning followed by a long die-away part. The plateau represents the well-known lag period (*21*) during which little or no coverage takes place. According to the curve, the lag period covered an interval of 3 to 5 days. The die-away feature is the same as that obtained by Carrel and DuNoüy and shows that the area covered per day continually decreased. It should be emphasized, however, that while this curve gives information pertaining to area, it shows nothing about the rate of growth or functioning of the cells at any point along the wound borders.

Values based on the breadth measurements (table 1) were plotted for each wound individually in figure 3, and the averages for six wounds (not shown in table 1) were plotted in figure 4.

Figure 4 shows that the points are distributed in a manner not greatly unlike those in figure 2. A smooth curve drawn through the points (dotted-line curve) also shows a short plateau at the beginning followed by a long die-away part. This curve, however, can be divided into three straight sections (solid lines). Since the points fit so closely to straight lines, it appears that epithelial coverage,

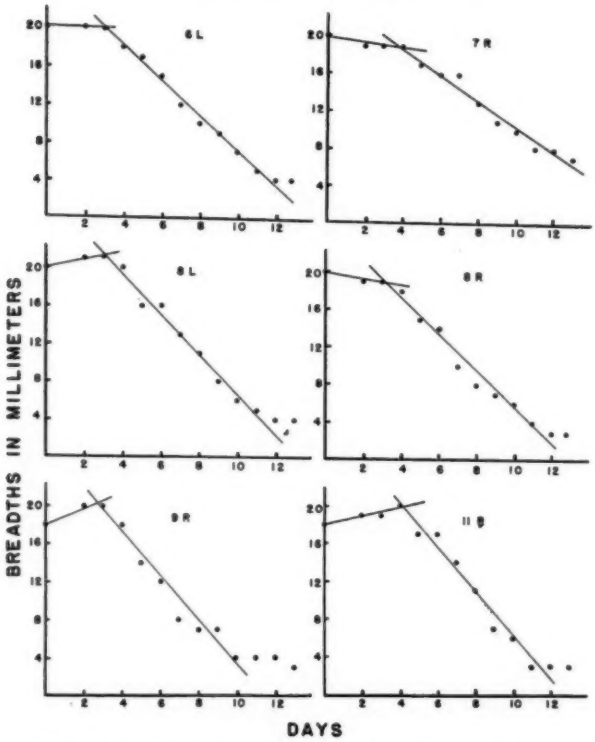

FIGURE 3.—Curves for individual wounds showing how the breadths vary with time.

as measured in terms of breadth, displayed three distinct phases of growth behavior: (1) A lag phase the same as indicated by the area curve; (2) a main-growth phase which covered most of the growth period; and (3) a phase of reduced growth which took place as the wounds were being finally closed. Since the second phase concerns the greater portion of the coverage process, it contains the most significant information regarding coverage rate.

The points as plotted, being derived from breadth measurements, pertain to exposed fields; accordingly, the slopes of the curves indicate the rates of lateral extension of the epithelium. Since the points follow closely a straight line course, it is clear that the rate of lateral growth remained essentially constant throughout the main healing period. In figure 3 these same features are as clearly manifested by the curves for the individual wounds as by the curve for the averages. It will be seen, however, that while the rate of lateral extension in each wound is essentially constant, it may differ in different wounds. Actual determinations for the different curves showed it to vary from —0.70 to —1.15.

The third phase of growth covers actually only a small portion of the healing process; and because it was so small, there was a tendency at first to overlook it as an artifact. However, since it appears in all of the curves for individual wounds which extended far enough to show it, it appears to be a real feature. We have, in fact, seen it clearly displayed in other wounds on the rabbit which have not been reported as yet. In a consideration of the nature of the coverage process, this phase is likely to have considerable significance. Since we are concerned

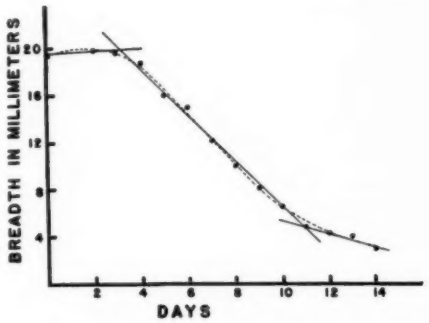

FIGURE 4.—Curves for averages of six wounds showing how the average breadth varies with time.

mainly with dosage rates and since this represents such a small portion of the coverage process, no further attention will be given to it.

These findings alone clarify much of the wound-measurement problem. Further, they are in agreement with the findings of Lumiére obtained so long ago as well as those of Howes obtained recently. So far as the rabbit's ear is concerned, they give quantitative confirmation of the fact that the rate of lateral extension (barring complications) takes place at a uniform rate and furnishes evidence that there is orderly demobilization of the healing forces as the exposed areas become covered. They also show that the repair activity throughout the main growth period is essentially the same at all points along the perimeter, irrespective of its length.

With this information at hand, it is possible to give a complete explanation of the statements of Carrel and Du Noüy that the rate of coverage continually decreases as the wound becomes smaller. Since the growth rate remains essentially the same at all points along the wound borders during the main growth phase and since the perimeter becomes continually shorter, the amount of area covered per unit time naturally decreases. Thus the statement by Carrel and Du Noüy has meaning only with respect to area and has no bearing on the question of growth capacity of cells or the repair rate in the tissues of wounds.

From these results, it is obvious geometrically (1) that for the main growth phase the rate of repair in terms of area varies directly with the wound perimeter, and (2) that the healing time is proportional to the breadth of the wound (i. e., for regular-shaped wounds, such as circles, ellipses, squares, rectangles, and parallelograms).

EXPERIMENT 2

If the foregoing findings are correct, the healing time of wounds of different sizes and shapes should be the same as long as the initial breadth is the same. In an experiment designed to test this assumption, wounds were prepared on the rabbit's ear as before, but with the following dimensions: 20 x 20 mm., 10 x 40 mm., and 10 x 10 mm. Wounds of the first and second types had the same areas, but the second had a breadth only half as large as the first. The second and third had equal breadths, but the area of the second was four times that of the third. Hence, if the healing time is proportional to the breadth, the second and third wounds should heal in the same time, and the first should take twice as long.

Five sets of wounds were prepared, and the values for a typical set were plotted (fig. 5). The solid circles are for the 10-x-10-mm. wounds, the open circles for the 10-x-40-mm., and the triangular ones

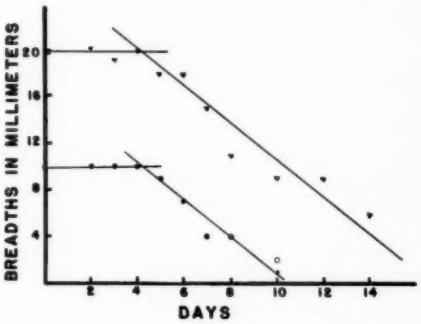

FIGURE 5.—Curves showing how breadth varies with time in epithelial wounds of different sizes and shapes. The triangular points represent values for wounds 20 x 20 mm. at the beginning whereas the open and closed circles represent values for wounds which were 10 x 10 mm. and 10 x 40 mm., respectively, at the beginning.

for the 20-x-20-mm. It is apparent that the wounds with equal breadths healed in the same time despite the large differences in area, whereas the one with the greater breadth took longer. It will be noted that the lag period and also the growth rates were essentially the same in each case, irrespective of the size or shape of the wounds. The findings in experiments 1 and 2, therefore, agree indicating that the assumption based upon geometry of wound shapes and the constancy of lateral extension is true.

IRREGULAR WOUNDS

Thus far only regular wounds (circles, ellipses, squares, rectangles, and parallelograms) have been discussed. What, then, is the situation when the wounds are triangular, crescentic, or otherwise irregular? If the line of growth is straight out from the borders, obviously the healing edges must meet at middle points in irregular-shaped wounds the same as in regular, and the last region to be covered is that farthest from any edge. In some wounds, however, the growth lines which bear on such a region do not approach from opposite directions. A triangular wound is a good example. In such a case the healing time cannot be related significantly to the breadth. The term "breadth," in fact, has very little meaning in connection with triangular areas.

If the line of growth at all points is straight out from all borders (that is, in a line perpendicular to a tangent at the border), it can be shown geometrically in the case of triangular wounds and in fact in wounds of any shape, that the diameter of the largest inscribed circle can be used in precisely the same manner as the breadth in squares, rectangles, parallelograms, or ellipses.[2] In the case of the figures just named, the breadth and diameter of the largest inscribed circle are identical. Based on the developments shown, time for complete healing in any shape of wound should be proportional to the diameter of the largest inscribed circle that can be placed over the wound without overlapping any borders (fig. 6). Likewise, the healing time of any arm or irregular projection of a wound will be proportional to the diameter of the largest inscribed circle for that arm. In practice, the healing time of any wound or any part of a wound can best be judged by estimating the diameter of the largest circle that can be placed over the area without overlapping the edges.

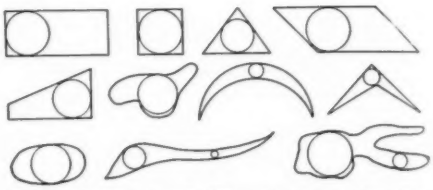

FIGURE 6.—Areas of different shapes showing how the largest inscribed circle is fitted.

DISCUSSION

As indicated at the beginning, most attempts at determining coverage or healing rates in surface wounds were based on measurements of areas. The steps involved were as follows: Formulas were devised for curves of the die-away type (hyperbolic or exponential functions) which characterized the healing process. Area measurements were made at intervals to establish a rate factor. This factor was inserted in the formulas and the healing time calculated. By such procedure the healing time could be predicted with fair accuracy.

There are, however, at least three objections to this method of determining healing time: (1) The procedure in every case involves the tedious task of measuring areas and the use of mathematical formulas; (2) the area approach may lead to false impressions, as has been indicated; (3) the value of the rate factor as determined on the area basis gives no information about the healing action of the cells or tissues. A high healing rate merely indicates that a large wound is under consideration and a low healing rate that a small one is being observed.

From our experiences it would seem that measurement of the breadth (or the diameter of the largest inscribed circle) of epithelial wounds is not only the simplest and easiest method of determin-

[2] Dr. H. W. Chalkley, of this Institute, suggested this application of the inscribed circle.

ing healing rates and healing times but also the most informative. Breadth can be measured without the aid of tracings, photographs, planimeters, etc. With calipers or a graded series of circles, measurements of breadth or diameter can be made easily. The rate of wound closure can likewise be determined simply and without the aid of mathematical formulas. It is necessary only to make a few measurements during the main growth phase, plot these on a graph, and draw the best straight line; the slope of the line gives the healing rate (millimeters per day). Furthermore, the healing rates in such a case characterize the growth capacity of the cells or tissues along the wound borders. If two growth curves, based on breadth or diameter measurements, differ in slope, it means that the functional activity of the cells involved in repair is different. By using growth curves obtained in this way, one can determine the healing rates in different wounds or in the same wounds at different times or under different conditions.

If one wishes to use a formula for calculating healing time, the simple one proposed by Lumiére is adequate. Lumiére pointed out that since the rate of healing is essentially constant when measured in terms of breadth, the rate V is equal to $\frac{L}{T}$, where L is the breadth at the beginning and T the time required for complete healing. Hence the healing time determined by the formula is $T = \frac{L}{V}$, the rate V being available as soon as the slope for the growth curve is established. Thus, the healing time can be determined within 24 to 48 hours after active growth starts.

It should be emphasized, however, that the breadth formula can be employed for predicting healing times only when the wound growth is not disturbed by complicating factors. For this reason, it has limited practical value. It should, nevertheless, be especially useful in connection with investigative work when the effect of various agents or treatments are being tested on healing rates, when there is a desire to study the healing capacities of the wound tissues, and when there is a need to separate the lag phase from the growth phase in wound-repair studies. Furthermore, breadth measurements should be useful when there is a need for obtaining graphic records of the influence of extraneous factors such as necrosis, infection, and inflammation.

SUMMARY AND CONCLUSIONS

Epithelial wounds were prepared on the rabbit's ear for the purpose of comparing different methods of measuring wound growth and also for studying wound behavior. Removal of skin was done in such a manner as to cause a minimum of disturbance to subcutaneous connective tissue and blood vessels. Wound repair in these cases consisted almost exclusively of epithelial coverage.

The coverage process was observed closely, and daily measurements were made of the length and breadth of the exposed areas. Daily photographs were also made of the individual wounds.

Analysis of breadth and area data showed that the breadth determinations were not only the simplest and easiest to make, but that they gave more useful and more important information.

On the basis of breadth measurements, the repair process was found to display three phases: (1) A lag phase lasting 3 to 5 days during which little or no coverage took place; (2) a phase of active growth lasting a longer period depending on the size of the wound; and (3) a phase of reduced growth near the end of the healing period.

During the phase of active growth the rate of lateral extension of the epithelial sheets was found to be essentially constant. Wounds having the same breadth were found to heal in the same time irrespective of size or shape.

It became apparent from geometrical considerations that the rate of epithelial repair in terms of area varied directly with the wound perimeter and that the healing time was proportional to the breadth in regular-shaped wounds.

It likewise became apparent that for irregular-shaped wounds, the diameter of the largest inscribed circle could be used in the same manner as the breadth in case of the regular-shaped wounds.

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INJECTION AND CLEARING METHOD FOR THE RABBIT'S EAR

By THELMA B. DUNN, *research fellow*, and ALBERT M. KESSEL, *senior medical technician, National Cancer Institute, National Institute of Health, United States Public Health Service*

An injection and clearing method for the study of the vascular structure in the rabbit's ear is presented because it is simple to follow, and the finished preparation is easily displayed or stored. It was devised to show the gradually developing effect of X-ray injury, and it can be used in any similar type of study.

White animals must be selected, and any experimental injury should be produced near the tip of the ear to avoid folds when the ear is mounted. Previously made tattoo marks may be used to outline the treated area as in the specimen shown in figure 1.

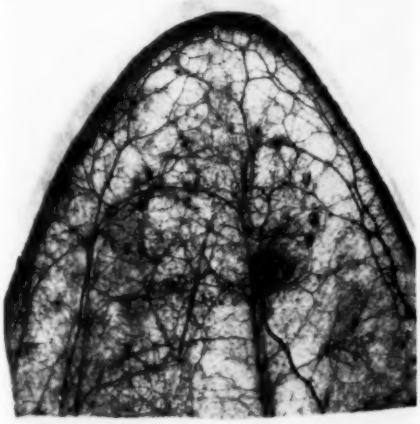

FIGURE 1.—Rabbit's ear injected, cleared, and mounted. Natural size.

The injection apparatus consists of a mercury manometer, a large test tube containing about 50 cc. of a mixture of 1 part india ink to 8 parts tap water, a 50-cc. syringe, and an 18-gage needle. The needle should be cut across near the tip and a small collar made by applying solder, which helps to hold the needle in place after it has been introduced into the artery. A two-hole rubber stopper is fitted to the test tube. Through one hole a short piece of glass tubing is passed, and this is attached by ¼-inch-bore rubber tubing to a **Y** tube which in turn is connected to the manometer and the syringe. Through the second hole a longer piece of tubing is inserted which will reach almost to the bottom of the tube, and this is connected by rubber tubing to the injection needle.

The ear is carefully shaved and the animal killed by any convenient method. The ear is then quickly severed at the base, and under the dissecting microscope the needle is introduced into the median artery for about a centimeter. Ink solution is then forced through the vessels until the whole ear is uniformly dark, when the larger veins at the base are clamped off, and a pressure of 200 mm. is maintained for 1 minute. Both arteries and veins are then clamped, and the ear is put into pure glycerin and loosely held between glass plates in a flat staining dish. On the second day the basal part of the ear may be trimmed away, and the part to be studied pressed out more smoothly. The ear is readjusted about once a day; the glycerin may be renewed if necessary. Clearing is usually complete in about a week. This technique is modified from the methods described by Winternitz, Thomas, and LeCompte,[1] and by Schlesinger,[2] and for more complete details, these sources should be consulted.

When clearing is complete, the specimen may be kept indefinitely immersed in glycerin, and removed, whenever it is desired, for study under the dissecting microscope, or it may be mounted by the method described.

A glycerin jelly is made as follows:

Gelatin	5 gm.
Distilled water	25 gm.
Glycerin	75 cc.
Carbolic acid	2 gm.

[1] WINTERNITZ, M. C., THOMAS, R. M., and LECOMPTE, P. N.: The biology of arteriosclerosis. Thomas, Springfield, Ill. (1938).

[2] SCHLESINGER, M. J.: An injection plus dissection study of coronary artery occlusions and anastomoses. Am. Heart J., 15: 528–568 (1938).

After mixing, it is boiled gently for 5 minutes with frequent stirring. It may then be stored indefinitely or used at once.

A glass plate 3¼ x 4 inches in size is placed on the bottom of a flat staining dish, which is then partly filled with the melted gelatin mixture. The specimen is put in place and covered by a second plate of the same size. Inclusion of air bubbles must be avoided. A 100-gm. weight is next put on the preparation, and the dish is set aside until the gelatin solidifies. After this the mounted specimen can be cut out of the dish with a knife. If it adheres to the bottom of the dish, it can be loosened by placing the dish on a warm plate, care being taken to prevent melting the entire mount. Excess gelatin on the outside is wiped off with warm water. The edges are sealed with ¾-inch scotch tape, which must be snugly applied. If it should be necessary to open a mount already sealed, this is easily done by removing the tape and placing the mount on a warm plate or in an incubator.

The mounted specimen is very convenient for study under the dissecting microscope, and if the mounting has been kept thin and the hair removed, it can be projected from a lantern or a microprojector and demonstrated to a large group. Some of our preparations have been projected from the lantern for 5 minutes without injury.

A satisfactory injection of the capillaries and veins is always obtained. The larger arteries are frequently empty but are easily identified because of fine ink particles adhering to the walls. This method is useful primarily for a comparative study, and it is often helpful to use the opposite ear as a control.

MULTIPLE TISSUE CARRIER FOR HISTOLOGIC TECHNIQUES[1]

By ALBERT M. KESSEL, *senior medical technician, National Cancer Institute, National Institute of Health, United States Public Health Service*

Technical procedures developed on a small scale offer possibilities for greater uniformity of performance and results and for considerable saving of time when extended to large-scale production. These possibilities have not been fully exploited in techniques of preparation of histologic sections. Devices for handling large numbers of slides have been devised for application of standardized staining techniques on a large scale. The transfer of tissues from one solution to another in the process leading up to embedding has continued to be time-consuming and subject to considerable differences in results from day to day and from one technician to another. These difficulties have been overcome to a considerable degree by the use of a device, described herein, for handling large numbers of pieces of tissue.

This device consists of a series of trays, each divided into compartments of variable size for segregation of tissue pieces into groups, suitably constructed to allow free circulation of fluids. The trays can be assembled in groups on the tray carrier, and when so assembled the complete group of trays fits into a beaker and can be transferred from one beaker to another as a single unit in the different steps of fixation and embedding.

CONSTRUCTION OF APPARATUS

The various parts must be constructed of a material that is resistant to all chemicals in which it is to be immersed. Stainless steel or chromium plating over ordinary steel has been used and found satisfactory for use with the solutions employed in dehydration and embedding. The construction of the various parts is as follows:

The tray carrier (fig. 1, *A*) consists of a rod 3/16 by 10 inches, one end of which is attached to the center of a round plate. The end of the rod is either threaded or riveted into the plate. The plate is 1/16 inch in thickness and 3⅝ inches in diameter. It has a number of holes ¼ inch in diameter.

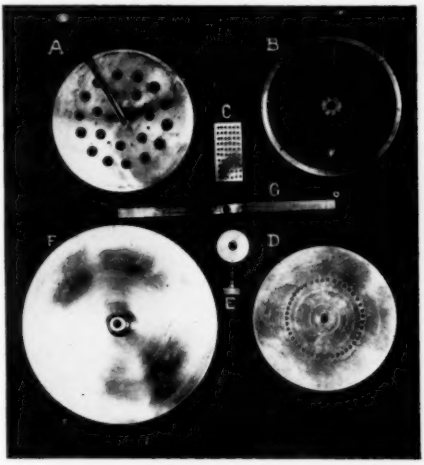

FIGURE 1.—Multiple tissue carrier. *A*, tray carrier; *B*, tissue tray; *C*, perforated plate; *D*, lid; *E*, cover retaining collar; *F*, cover; and *G*, carrier holder.

All the tissue trays (fig. 1, *B*) are similar in over-all size and design. The frame of the tray is a ring prepared from tubular metal, 3½ inches in external diameter, 3¼ inches in internal diameter, and ¾ inch in height. There are eight equally spaced vertical grooves on the inner surface of the ring. Each groove has a clearance of 3/64 inch. A fine wire mesh, soldered to the lower side of the ring, converts the ring into a tray. The tray is subdivided into eight compartments by eight partitions which run from the grooves on the inner surface of the ring toward the center. Here they fit into similar grooves on the outer surface of a centrally placed, small hub ½ inch in external diameter and ¾ inch in height. This hub has a central hole 3/16 inch in diameter. The lower surface of the hub is soldered to the tray floor or upper surface of the wire meshwork. Each of the partitions that subdivide the tray into compartments is a perforated plate (fig. 1, *C*) 1½ inches in length and

[1] Covered by U. S. Patent No. 2,302,698, issued November 24, 1942.

¾ of an inch in height and somewhat less than 1/32 of an inch thick. Two of the partitions are soldered in place so that the tray is divided into two permanent compartments of equal size. This arrangement also lends support to the central hub. The remaining six partitions are removable. When they are all in place, they divide the tray into eight compartments. Numbers are stamped on the rim of the tray to identify each compartment.

The lid for the uppermost tray (fig. 1, *D*) is a circular plate 3⅝ inches in diameter and 1/16 inch in thickness. It has a central perforation measuring 3/16 inch, and in addition a number of holes each measuring up to 1/16 inch in diameter.

The cover-retaining collar (fig. 1, *E*) consists of a disk ⅞ inch in diameter and ¾ inch in height. There is a hole 3/16 inch in diameter through the center. A threaded thumbscrew extends through the lateral surface to the central hole.

The cover for the container of fluid (fig. 1, *F*) consists of a metal plate 5 inches in diameter and 1/16 inch in thickness, with a central guide attached to the upper surface of the cover. This guide is a disk ½ inch in width and ½ inch in height. In the center of guide and cover is a hole 3/16 inch in diameter so that they can be fitted over the rod of figure 1, *A*.

The carrier holder (fig. 1, *G*) is a rectangular bar 5½ inches in length and ¼ by ¼ inch in cross section in the center of which is a hole 3/16 inch in diameter.

OPERATION OF DEVICE

The carrier holder is used to lower or raise successive trays near the surface of the fluid in a beaker of 1-l. capacity for convenience during loading and unloading of the carrier. In this process the carrier holder is used in place of the lid shown in figure 2. The cover and the cover-retaining collar serve to cover the beaker and minimize evaporation of fluid and to adjust the height of the trays in the beaker so that the lowermost tray does not rest upon the bottom of the beaker, thereby facilitating circulation of the fluid (fig. 2). A record is kept of the number of the compartment in which tissues are placed for identification. The carrier can be transferred from one fluid to the next manually, or it can be used in conjunction with a device for automatic changing.

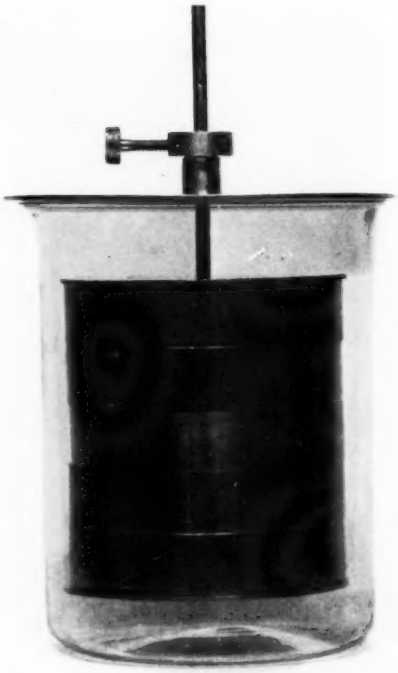

FIGURE 2.—The multiple tissue carrier assembled.

In addition to considerable saving of time, the carrier makes possible the exposure, under uniform conditions, of large numbers of pieces of tissue to various solutions in the process of dehydration and infiltration. This is especially valuable if the degree of shrinkage of the tissue is an important consideration in a subsequent comparative study of the sections.

PRODUCTION OF MALIGNANCY IN VITRO. VII. METABOLISM AND BIOTIN VITAMER CONTENT OF TUMORS PRODUCED

By DEAN BURK, *senior chemist*, WILTON R. EARLE, *senior cytologist*, and RICHARD J. WINZLER, *research fellow*, with the technical assistance of DORIS F. MACNEARY, MARIE HESSELBACH, EMMA SHELTON, and EDWARD L. SCHILLING, *National Cancer Institute, National Institute of Health, United States Public Health Service*

This paper presents a metabolic analysis of transplanted mouse sarcomas (*1-5*) derived from mouse subcutaneous fibroblasts that had been treated in tissue culture with 20-methylcholanthrene [1] for varying periods of time. The first objective was to determine whether these tumors, the malignancy of which had been induced in vitro, possessed that tissue-slice metabolism now commonly regarded by Burk (*6-13*) as characteristic of malignant tumors of in vivo origin, spontaneous or induced. A second objective was to observe any quantitative or qualitative metabolic differences that might exist among the tumors, for correlation with differences already noted in regard to morphology, histology, transplantability, metastasis, or growth rate. Finally, measurements of tumor vitamin content were made with respect to biotin and miotin, since these substances have been reported to have a fundamental coenzyme action in fermentation, respiration, nitrogen assimilation, or growth of microorganisms or animals (*14-19*), and to be involved in the problem of tumor development (*19, 20, 21*).

MATERIALS AND METHODS

Seven strains of induced tumors, HW, D, H, J, L, N, and O, were studied. Earle and coworkers (*1-5*) have described their characteristics and their origin from cells of a primary strain of fibroblasts originally grown from a clump of subcutaneous tissue of a young strain C3H male mouse. Strains H, J, L, N, and O all received 1.0 γ of methylcholanthrene per cubic centimeter of culture fluid, for different intervals of time as indicated in table 1, after which the cultures were carried on for various intervals without addition of carcinogen to the culture fluid. At the time the present series of metabolic studies was started (April 1943), the last culture (strain O) to have carcinogen removed from the medium had been carried in vitro without addition of methylcholanthrene for 49 days, and the other strains N, L, J, and H for 123, 221, 289, and 335 days, respectively. All strains had been further carried through seven or more generations of subinoculation in mice. The HW and D cultures never received any deliberate treatment with carcinogen, but about June 1942, after cultivation in vitro for approximately 20 months, they showed modifications of the same type as did the other strains, but of lesser degree, suggesting that a trace contamination with the carcinogen had occurred. All cultures were grown in a completely heterologous medium. On injection into C3H mice all seven strains of cells gave rise to tumors, with variable percentages of takes (table 1). The tumor strains were carried on by subinoculation of C3H mice. The transplantability of tumors once formed has been practically 100 percent for all strains.

Complete details regarding the methods and procedure for obtaining the metabolic values, together with considerations of many special technical problems, including sources of error and interpretation of replicate variability, have been described in this Journal (*9, 10*). All manometric measurements were made at 38° C. All Q values are in terms of cubic millimeters/milligrams initial aliquot dry weight/hour, whereby 1 mm.^{3} = 1/22400000 mol. If Q values were based upon final rather than initial dry weights of tissue, taken at the end of experimental runs after considerable loss of cell substance into the medium, as was done in many of the earlier tissue-metabolism studies, such Q values would be 50 to 100 percent higher, but less generally satisfactory (*9, p. 227*). The constant-volume Warburg manometer was used to measure anaerobic (lactic) acid production ($Q^{N_2}{}_A$) in bicarbonate-buffered medium,

[1] Hereinafter referred to as methylcholanthrene.

TABLE 1.—*Anaerobic glycolysis at pH 7.3 and pH 7.0 of transplanted mouse sarcomas originally derived from mouse fibroblast cells treated in tissue culture with 20-methylcholanthrene for varying periods of time*

Strain	Treatment with 20-methylcholanthrene	Mice inoculated with tissue cultures	Tumors obtained	Anaerobic glycolysis at pH 7.3								Anaerobic glycolysis at pH 7.0								
				Date of analysis	Tumor No.	Transplant No.	Age of tumor	Initial dry weight	$^1QN_2{}_A$			Date of analysis	Tumor No.	Transplant No.	Age of tumor	Initial dry weight	$^1QN_2{}_A$			Difference between mean $QN_2{}_A$ values at pH 7.3 and pH 7.0	Probability, P
									Individual determinations	Mean	Standard deviation						Individual determinations	Mean	Standard deviation		
	Days	*No.*	*Pct.*	*1943*			*Days*	*Pct.*				*1943*			*Days*	*Pct.*					
HW	0	39	7.7	Apr. 29	HGW/AH17	8	16	16.9	18.0; 17.1	16.5	1.66	June 25	HGW/AL54	12	15	14.6	12.8; 12.1; 11.9	13.5	1.26	3.0	<0.01
					HGW/AH18	8	16	15.5	17.7				HGW/AL55	12	15	16.0	16.2; 14.9; 14.9				
					HGW/AQ54	17	14	14.2	15.2; 14.8				HGW/AL49	12	18	13.6	15.1; 13.3;				
												June 28	HGW/AL53	12	18	14.8	13.7; 13.3; 13.0				
													HGW/AL56	12	18	14.3	12.8; 12.6; 12.2				
												Sept. 18	HGW/AL50	12	18	14.9	14.5; 12.3				
													HGW/AQ54	17	14	14.2	14.6; 13.0				
D	0	83	86	Apr. 17	D/AG17	7	19	16.6	20.0; 18.2; 16.2	18.3	2.12	June 25	D/AL7	12	15	17.0	16.9; 14.1	14.6	1.89	3.7	<.01
				Apr. 29	D/AH20	8	12	18.0	20.7; 19.4; 19.3				D/AL6	12	15	16.8	15.6; 14.0; 13.5				
				June 28	D/AL3	12	18	16.2	18.3; 16.7; 14.4				D/AL5	12	15	19.4	14.4; 13.5				
				Sept. 18	D/AQ6	17	14	18.0	21.4; 16.7			June 28	D/AL8	12	18	16.6	15.8; 14.7; 13.5				
													D/AL3	12	18	16.2	16.1; 12.9; 12.4				
												Sept. 18	D/AQ6	17	14	18.0	16.6; 15.8				
H	6	65	72	Apr. 29	HJ/DG19	7	19	16.5	18.2; 17.0; 16.6	17.4	1.35	June 25	HJ/DK9	11	15	16.5	13.8; 13.3; 12.8	14.2	1.90	3.2	<.01
				June 10	HJ/DJ11	10	14	15.7	17.7; 16.3; 15.6			June 28	HJ/DJ14	11	18	15.6	13.9; 12.6				
				Sept. 18	HJ/DP12	16	14	15.3	19.3; 18.6			Sept. 18	HJ/DP12	16	14	15.3	16.9; 16.3				
J	32	45	20	Apr. 29	JG/AH5	8	16	15.0	17.0; 16.0; 15.1	16.2	1.51	June 25	JG/AL18	12	15	16.1	15.2; 12.5; 11.6	14.3	2.08	1.9	.06
				June 10	JG/AG20	11	14	16.0	17.1; 17.1; 15.3				JG/AL22	12	15	15.9	16.2; 13.7; 12.8				
				Sept. 18	JG/AQ24	17	14	15.9	19.0; 12.8				JG/AL19	12	15	16.3	15.1; 12.3				
												Sept. 18	JG/AQ24	17	14	15.9	17.0; 16.8				
L	111	54	46	Apr. 17	LF/AG2	7	19	16.8	14.4; 12.8; 10.0	16.4	2.61	June 25	LF/AL30	12	15	16.4	18.0; 14.8; 13.1	15.3	2.20	1.1	.35
				Apr. 29	LF/AH4	8	12	16.7	18.8; 18.1; 17.3			Sept. 18	LF/AQ25	17	14	15.5	16.3; 14.5				
					LF/AK25	11	14	16.5	16.7; 15.5; 15.0												
				June 10	LF/AK26	11	14	15.7	17.4; 16.8; 14.7												
					LF/AK28	11	14	14.6	21.0; 17.4; 17.0												
				Sept. 18	LF/AQ25	17	14	15.5	19.9; 16.1												
N	184	66	7.6	Apr. 29	NF/AH16	8	16	15.6	19.8; 16.7; 15.1	17.0	1.79	June 25	NF/AL38	12	15	15.5	13.4; 11.4; 10.3	15.1	2.75	1.9	.05
				June 28	NF/AL34	12	18	15.2	17.3; 15.9; 15.8				NF/AL39	12	15	12.3	19.2; 18.7; 18.3				
				Sept. 18	NF/AQ35	17	14	15.8	19.2; 16.6				NF/AL37	12	18	15.4	14.0; 13.2; 12.5				
												June 28	NF/AL34	12	18	15.4	17.8; 16.4; 15.4				
													NF/AL33	12	18	15.5	14.9; 12.5				
												Sept. 18	NF/AQ35	17	14	15.8	18.8; 14.6				
O	406	26	7.6	Apr. 17	OA/AG11	7	19	18.2	17.4; 15.8; 14.6	18.0	2.45	June 25	OA/AL42	12	15	18.4	14.9; 13.7; 13.3	15.9	3.06	2.1	.20
				Apr. 29	OA/AH11	8	12	17.5	22.0; 20.5; 15.9			Sept. 18	OA/AQ41	17	14	16.2	20.0; 17.5				
				June 10	OA/AK41	11	14	19.0	18.5; 17.4; 14.9												
					OA/AK45	11	14	18.0	19.1; 17.8; 17.2												
				Sept. 18	OA/AQ41	17	14	16.2	21.7; 19.4												

$^1QN_2{}_A$ values in cubic millimeters/milligram initial aliquot dry weight/hour.

and also oxygen consumption (Q_{O_2}) in phosphate-buffered medium (with absorption by KOH in vessel inset of CO_2 produced). The constant-volume differential Summerson manometer was used to measure aerobic (largely lactic) acid production ($Q^{O_2}{}_A$), respiratory CO_2 production (Q_{CO_2}), oxygen consumption (Q_{O_2}), and respiratory quotient (R.Q.= Q_{CO_2}/Q_{O_2}), all in bicarbonate-buffered medium. The basic medium was Warburg-Okamoto-Ringer's solution containing 0.2 percent glucose and either 0.013 M sodium phosphate or 0.025 M sodium bicarbonate, ordinarily at an initial pH of 7.4. This pH was established in the bicarbonate medium by the presence of 5 percent CO_2 in the gas phase. Experimental runs of 1 to 3 hours resulted in final pH values of about 7.2, with an over-all average during the runs that was taken to be 7.3. A pH of 7.0, as reported in table 1, was obtained by titrating the medium with HCl to give about the same color with phenol red as was given at pH 7.4 by cresol red, with final checking by means of the glass electrode. Little or no change in pH was observed in the experimental runs at pH 7.0. The values 7.3 and 7.0 in table 1 involve a maximum deviation of $\pm$ 0.1 pH unit in any individual experimental vessel.

Biotin and miotin were measured by the yeast growth method at 30° C., in slanted, stationary test tubes containing 6 cc. of mineral salts-ammonium sulfate-sucrose-casein hydrolysate-vitamin medium, in the absence and presence of (partially purified) avidin (ca. 0.002 units/cubic centimeter, an excess). Total biotin vitamer content was represented by the growth in the absence of avidin, miotin content by the growth in the presence of excess avidin, and biotin content by the difference between the total biotin vitamer content and the miotin content. The tissues were prepared for analysis by autoclaving for 2 hours in 5 percent sulfuric acid and neutralizing to the pH of the culture medium, 4.5.

EXPERIMENTAL PROCEDURE

STANDARD METABOLISM

Tables 1 and 2 report all metabolic data taken in bicarbonate medium. The tumors are arranged in order of increasing histologic change, corresponding to increasing length of exposure of the original tissue cultures to methylcholanthrene. In accord with various metabolic criteria of malignancy set up earlier (*6, pp. 242; 7, pp. 440-446*), it is seen that at pH 7.3 the tumors have a very high anaerobic glycolysis ($Q^{N_2}{}_A = 15$ to 19), a considerable aerobic glycolysis ($Q^{O_2}{}_A = 3$ to 6), a respiratory quotient definitely below unity (R. Q. about 0.85), a moderate oxygen consumption ($Q_{O_2} = 6$ to 8), a considerable absolute Pasteur effect ($Q^{N_2}{}_A - Q^{O_2}{}_A = 10$ to 12), a percentage Pasteur effect well below 100 (100 ($Q^{N_2}{}_A - Q^{O_2}{}_A$) / $Q^{N_2}{}_A$=ca. 70), a Meyerhof oxidation quotient several times unity ($3(Q^{N_2}{}_A - Q^{O_2}{}_A) / Q_{O_2} = 4.5$), an extent quotient ($3(Q^{N_2}{}_A / Q_{O_2})$ greater than 6, and a positive fermentation excess ($U = Q^{N_2}{}_A - 2Q_{O_2} = 1$ to 6); the ranges of parenthetical Q values cited are meant to include the great majority of values, not necessarily the extreme limits in every case. A final criterion, based on stimulation of oxygen consumption by succinate and paraphenylenediamine, will be considered in connection with table 3. No tumor or class of tumors is known to us that more satisfactorily meets all of these metabolic criteria of

TABLE 2.—*Aerobic metabolism[1] of tumors in Ringer–bicarbonate–glucose medium*

Strain	Tumor No.	Anaerobic acid formation[2] ($Q^{N_2}{}_A$)	Aerobic acid formation ($Q^{O_2}{}_A$)	Carbon dioxide production (Q_{CO_2})	Oxygen consumption (Q_{O_2})	Respiratory quotient (Q_{CO_2}/Q_{O_2})
D	D/AG17	20.0	6.7	5.7	6.9	0.83
		18.2	3.0	5.1	5.7	.89
		16.2				
L	LF/AG7	14.4	.30			
		12.8	4.0	6.7	7.6	.88
		10.0				
O	OA/AG16	17.4	6.4	6.0	6.9	.87
		15.8	6.5	7.2	8.5	.85
		14.6				
Average		15.5	4.9	6.1	7.1	.86

[1] Q values in terms of cubic millimeters/milligrams initial dry weight/hour. Summerson constant-volume differential manometers employed.
[2] Data from table 1.

malignancy than the present series, in which the malignancy was induced in vitro.

As already mentioned, every individual Q value measured has been reported, in order to give any analysis of the data that might be made as broad a statistical basis as possible. We have not been able to note any significant and consistent influence of transplant number, age of tumor, or initial percentage dry weight, on the anaerobic glycolysis of either individual tumors or strains. An interesting differential effect of pH has been noted among the strains, however, that has suggested a promising line of investigation now in progress. As indicated in table 1 and figure 1, lowering the pH from 7.3 to 7.0 decreased the average $Q^{N_2}{}_A$ values by 3–4 units, with a high degree of significance ($P < 0.01$, or more than 100 chances to 1), in the case of strains HW, D, and H, strains which in tissue culture received methylcholanthrene for no more than 6 days. In the case of strains J, L, N, and O, which in tissue culture received methylcholanthrene for 32 to 406 days and which showed a high degree of morphologic change, the average $Q^{N_2}{}_A$ decreased between pH 7.3 and 7.0 by only 1 to 2 units ($P = 0.05$ to 0.35). The statistical analysis indicates that the results in the latter group of strains could have occurred by chance alone, in the sense that differences of the magnitude concerned are too small to be established statistically by the methods and numbers of observations employed. The possibility that small but real differences might in fact exist cannot be ruled out. In view of the general observation that glycolysis of tissues ordinarily decreases with pH below serum pH levels, it is likely that with a somewhat lower pH value than 7.0 statistically significant decreases in $Q^{N_2}{}_A$ could be established with the methods and numbers of observations employed, and that the present values at pH 7.0 would fall about the curves of decreasing glycolytic function in true chance fashion.

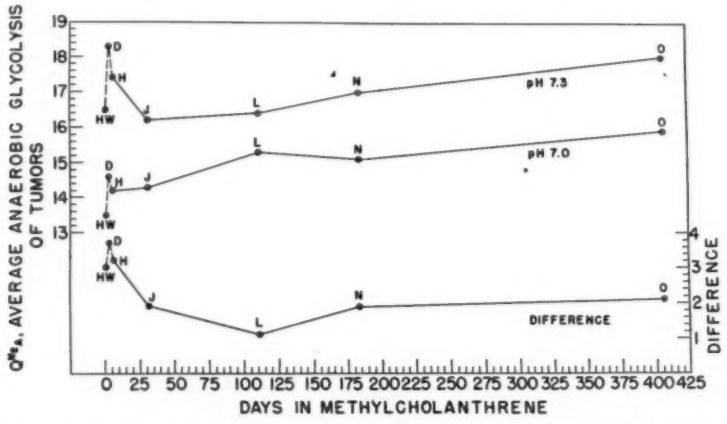

FIGURE 1.—Influence of pH and strain upon anaerobic glycolysis of tumors derived from tissue cultures treated with 20-methylcholanthrene for varying periods of time. Strain D points are located halfway between the ordinate and strain H points on the abscissa, and strain HW points halfway between the ordinate and the strain D points on the abscissa.

The standard deviations in table 1 are calculated with reference to total variance by assigning, in accordance with previous experience (*9, 10*), equal statistical value to each tissue-slice determination, which is the unit of experimental measurement ($S.D. = [\,(\Sigma y^2/n - \bar{y}^{-2})n/(n-1)\,]^{\frac{1}{2}}$, where $y = Q^{N_2}{}_A$. It does not seem desirable for the present to calculate any partial variance contributed to the total variance by factors such as numbers of tumors involved, age of

tumor, transplant number, percent dry weight, date, or other unreported factors that might be ascertainable. One of the most interesting of such factors would be the variability between tumors, but the data available are scarcely adequate for satisfactory analysis, and furthermore much of the observed variation between tumors is due to the same experimental factors that caused random variation among replicates of a given tumor. In this latter connection, analysis of the rather typical D group of 11 determinations on 4 tumors at pH 7.3 showed that the variation between tumors was not significantly greater than the variation within tumor replicates ($F = 1.55$, $P > 0.05$, where F would have to be 4.55 for P to be 0.05). An approximate study of the variation between tumors might be made from the data of table 1, but it would be greatly preferable to have data with many more replicates per tumor, or more accurate measurements, and, if feasible, a closer correlation with histological analysis. It would therefore seem best to postpone a solution of this secondary statistical problem, which is distinct from the statistical problem treated in table 1, where the individual determination and not the tumor is the statistic unit of interest, with respect to ultimate strain difference or strain grouping difference. Both problems, indeed, might be treated with great advantage by an entirely different statistical mode of analysis involving the control chart method [2] (*23*).

If the three mean differences at pH 7.3 and 7.0 in the HW-D-H group are compared statistically with the four mean differences in the J-L-N-O group, the average mean differences for the two groups are 3.3 (S. D., 0.44) and 1.75 (S. D., 0.30), respectively, with a difference between the group averages of 1.55 ± 0.11. This difference is 14 times its standard error ($P < 0.01$).

Experiments are in progress to see if this statistically significant distinction between the first three and the last four strains cannot be greatly increased at considerably lower pH values, obtained by either titrating the medium with strong acid or with CO_2 gas pressures higher than 5 percent, with due consideration of bicarbonate levels. Such a differentiation and gradation of pH effect would be especially significant with the present series of tumors, which were all derived from the same initial normal cells. It is quite possible that quantitative metabolic (glycolytic) distinctions might be greatly sharpened at say pH 6.3 to 6.5 as compared with 7.3, not only with the present series of tumors, and among tumors generally, but between tumors and those normal tissues (e. g., retina, kidney medulla, mucosa, and many growing tissues) which have a high glycolysis at about pH 7.3 to 7.4 and which appear to resemble tumors in this aspect of metabolism. Although the pH of arterial blood, 7.3 to 7.4, has usually been employed in tissue-metabolism studies heretofore, it is probable that a lower pH often obtains in the intercellular tissue fluids in vivo, especially in tumors and other glycolyzing tissues. Thus Voegtlin et al. (*24*) and Kahler and Robertson (*25*) found that the pH of tumors measured in vivo with a glass electrode fell to as low as 6.3 to 6.5, especially when sugar was given. Comparative glycolytic tissue-slice studies with various tumors and normal tissues in this pH range are therefore highly desirable and are in process of being carried out.

Although at any given pH the average glycolytic values for the different strains are more notable for their quantitative similarity than for their difference (which was not the case with a series of chicken tumors studied (*9*)), it does seem possible, on the basis of the statistical analysis already given, to conclude that the curves given in figure 1 do represent significant trends, probably of a curvilinear nature. The curves for pH 7.3 and 7.0, and also the curve for the difference between pH 7.3 and 7.0, resemble in certain respects curves previously obtained (*3, 4*) and reproduced in figure 2, in regard to percentage of inoculation takes (tissue-culture cells to host animal) and to rate of cell growth. There is some tendency for the D strain to give peak values, the J strain to give minimum values, the L strain to give a secondary

[2] Deming, W. E.: Private communication.

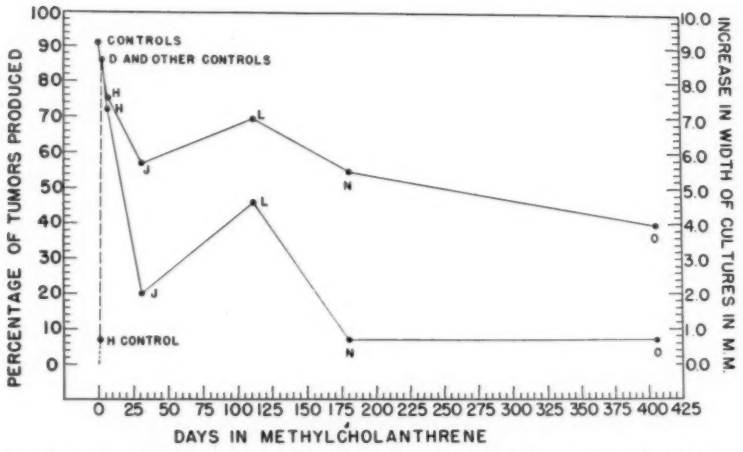

FIGURE 2.—Inoculation response (lower curve) and growth rate (upper curve) of tissue culture strains treated with 20-methylcholanthrene for varying periods of time.

peak, and for a slightly rising value between N and O. The basic reasons for these trends cannot even be guessed at present, but it seems worth while to call attention to them at this time. The average curve for pH 7.3, based on all values, was rather closely approximated by curves based upon average results obtained on any one of the different days on which observations were made, April 17, April 29, June 10, June 18, or September 18.

STIMULATION OF RESPIRATION BY SUCCINATE OR PARAPHENYLENEDIAMINE (OXIDATION RESERVE)

Salter, Craig, and coworkers (*26*, *27*, *28*) investigated extensively the response of normal and cancer tissues to the addition of succinate or paraphenylenediamine and concluded that the percentage stimulation of oxygen consumption by these agents was markedly greater in normal than in tumor tissues. Examination [3] of many scores of normal, embryonic, and tumor tissues in rabbits, chickens, and rats has yielded results that are in general accord with those of Salter, Craig, and coworkers. In our observations over 95 percent of the tumors examined gave a stimulation of Q_{O_2} of less than 100 to 125 percent, and the great bulk of them less than 60 percent, the response being almost invariably smaller with succinate than with paraphenylenediamine. On the other hand, 95 percent of all normal and embryonic tissues gave stimulations of over 100 percent, usually several hundred percent, and not infrequently over 1,000 percent. The observations of Salter, Craig, and coworkers, as well as our own,[4] are readily understandable on a basis of the low succinic oxidase, cytochrome, and cytochrome oxidase content generally observed in most tumors. The extent of response to succinate and/or paraphenylenediamine might well be a useful adjunct to morphologic studies for establishing the existence of a neoplastic state, in certain cases. Benign tumors have in our limited experience fallen in with the class of malignant tumors rather than with the class of normal or embryonic tissues.

Table 3 shows that all the tumors under study here give low responses of Q_{O_2} to addition of succinate or paraphenylenediamine. The positive stimulation by succinate is considerably less than that by paraphenylenediamine, and almost negligible within the limits of experimental significance. No important stimulation difference between the strains occurred in the present series of observations, except that strains L and J were

[3] To be reported elsewhere.

[4] To be reported elsewhere.

TABLE 3.—*Oxygen consumption (Q_{O_2}) of tumors in Ringer-phosphate-glucose medium in the presence and absence of succinate and paraphenylenediamine*

Strain	Tumor No.	Tumor age	Date	Control (H_2O)			Succinate			Paraphenylenediamine		
				−[1]	+[2]	Increase	−[1]	+[2]	Increase	−[1]	+[2]	Increase
		Days	*1943*			*Pct.*			*Pct.*			*Pct.*
HW	HGW/AH17	16	Apr. 29				6.7	7.3	9	5.6	7.7	37
										5.9	8.6	46
D	D/AG17	19	Apr. 17	8.3	7.6	−8	7.9	8.7	10	8.4	9.9	18
							6.0	5.9	−2	8.4	9.9	18
							8.1	8.8	9	10.0	13.2	32
	D/AH20	12	Apr. 29				9.1	10.1	11	9.4	11.6	23
										8.4	12.4	47
H	HJ/DG19	17	Apr. 29				8.8	9.8	12	8.5	10.9	28
										8.1	9.8	21
J	JG/AH5	16	Apr. 29				7.3	9.1	25	7.9	10.2	29
										7.2	9.3	29
L	LF/AG2	19	Apr. 17	6.8	7.3	7	7.2	8.2	14	6.8	11.1	63
							7.1	8.2	14			
	LF/AG7	19	Apr. 17				6.3	7.3	16	6.5	8.8	35
	LF/AH4	12	Apr. 29				6.0	7.2	20	5.7	8.8	39
										9.0	12.0	33
N	NF/AH16	16	Apr. 29				9.4	11.3	20	7.6	11.1	46
O	OA/AG11	19	Apr. 17	8.7	8.1	−7	7.9	8.2	4	8.2	9.8	20
							8.1	8.6	6	8.7	10.6	22
	OA/AG16	19	Apr. 17				8.1	9.1	12	8.2	9.8	20
	OA/AH11	12	Apr. 29							6.2	10.6	72

[1] Values based on readings during 1 hour before adding H_2O, succinate or paraphenylenediamine.
[2] Values based on reading during 1 hour after succinate was added by tipping from vessel side arm to bring its concentration up to ca. 0.02M; paraphenylenediamine added as 1 mg. solid per cubic centimeter from side arm.

perhaps higher than any of the others. Further work with a great many more determinations might make some definite strain distinction possible.

BIOTIN VITAMER ANALYSIS OF TUMORS

Aliquot samples of the tissue slices reported in tables 1 to 3 were analyzed for biotin and miotin (*19*). The results are given in table 4. No experimentally significant differences in biotin or miotin content were found among the seven strains, nor were the results notably affected by the size of the tumor pieces analyzed or by whether the pieces were dried or used fresh for the analyses. The average biotin content of about 0.6 micrograms per gram dry weight lies in the upper range of biotin values found by us or reported by others for tumors generally, and in the middle of the range for tissues generally. The miotin content, percent and absolute, is higher than that of practically any of a great variety of normal adult and embryonic tissues that we have examined, and is in the upper, though not extreme, range of values found for a variety of tumors. In this connection, it may be said that our results [5] show that tumors and also other growing tissues tend to have considerably higher absolute and percentage miotin values than do normal adult tissues, although there were definite exceptions to this generality.

SUMMARY

Seven strains of transplanted sarcomas, derived from mouse subcutaneous fibroblasts which had been subjected to various dosages of 20-methylcholanthrene in tissue culture, were found to possess a metabolism highly characteristic of malignant tumors generally, with respect to all criteria tested, including anaerobic glycolysis, aerobic glycolysis, respiratory quotient, oxygen consumption, oxidation reserve (increase in oxygen consumption on addition of succinate or paraphenylenediamine), Pasteur effect, Meyerhof

[5] To be reported elsewhere.

quotient, extent quotient, and fermentation excess. The biotin and miotin contents of these tumors of in vitro origin were likewise typical of values found in malignant tumors of in vivo origin, whether spontaneous or induced.

The metabolism of the seven strains of tumors was qualitatively and quantitatively very similar. Analysis of the data indicated, however, a rough, second-order correlation between minor differences in the anaerobic glycolysis values of the tumors and previously reported differences in growth rates and inoculation responses of the original tissue cultural strains. Lowering the pH of the medium containing the tumor tissue slices caused a considerably greater decrease in anaerobic glycolysis in the case of the three least altered strains, HW, D, and H, as compared with the four most altered tumor strains, J, L, N, and O. This sug-

TABLE 4.—*Biotin vitamer analysis of tumors* [1]

Strain	Tumor No.	Total per gram dry weight	Biotin per gram dry weight	Miotin per gram dry weight		Remarks
		Micrograms	*Micrograms*	*Micrograms*	*Percent*	
HW	HGW/AH17	0.52	0.47	0.05	10	Undried.
		.56	.52	.04	7	
	HGW/AH18	.71	.66	.05	7	Undried.
		.56	.51	.05	9	Undried chunk.
		.57	.52	.05	9	
		.65	.60	.05	7	
Average		.60	.55	.05	8	
D	D/AJ17	.80	.70	.10	12	
		.80	.77	.03	4	
		.80	.75	.05	6	Undried.
	D/AH20	.60	.56	.04	7	
Average		.75	.70	.06	7	
H	HJ/DJ19	.66	.62	.04	6	
		.58	.54	.04	7	Large chunks.
		.65	.61	.045	7	Small thin pieces.
		.63	.59	.04	6	Large thick pieces.
		.68	.64	.045	7	Chunk.
		.61	.58	.035	6	Large pieces undried.
Average		.64	.60	.04	7	
J	JG/AH5	.64	.57	.07	11	
		.80	.72	.08	10	
Average		.72	.65	.08	11	
L	LF/AG2	.60	.57	.03	5	
	LF/AG7	.65	.61	.05	8	
		.55	.52	.03	5	Undried.
	LF/AH4	.63	.59	.04	6	
		.54	.50	.04	7	
Average		.59	.56	.04	6	
N	NF/AH16	.54	.48	.06	11	Undried.
		.45	.41	.04	8	
		.58	.54	.045	8	
Average		.52	.48	.048	9	
O	OA/AG11	.45	.42	.03	6	
		.60	.54	.06	10	
	OA/AH11	.68	.65	.035	5	
	OA/Ak45	.69	.67	.03	4	
Average		.61	.57	.04	6	
Grand average		.63	.59	.047	7.4	

[1] Except as otherwise indicated in the final column, all analyses were made on tissue slices that were aliquot samples of those employed for metabolic studies in tables 1 to 3, after drying overnight at 65°–75° C. The percentage dry weights of the undried samples were obtained from aliquot dried samples. Values for miotin are for convenience expressed as equivalent to biotin, assuming equal molecular weight and activity (*19*).

gests that a more specific correlation between degree of malignancy and ability to glycolyze might be obtained at pH values considerably below that of blood, but closer to those that probably prevail within the tumor intercellularly.

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CHROMOSOMAL CHANGES IN EPIDERMAL CARCINOGENESIS [1]

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Progress made at the Barnard Hospital by several investigators in the integration of changes that occur in epidermal carcinogenesis has recently been summarized (*1*). It is clear that treatment of mouse epidermis with 0.6 percent methylcholanthrene in benzene results in the prompt establishment of a new kind of chemical equilibrium which is maintained without much change until some cells demonstrate by their altered behavior that they have become malignant. Because malignancy once developed seems to be a permanent attribute of a cellular population and also for other obvious reasons, it is important to determine the influence of carcinogen on nuclei. The present paper describes an attempt to discover whether the doubling and quadrupling in size of chromosomes, reported in other cancers (*2, 3, 4*), occur in this series, and, if so, their coincidence or lack of coincidence with other alterations both chemical and structural.

MATERIALS AND METHODS

A special series of methylcholanthrene-treated New Buffalo mice was prepared by means of the same technique of treatment as that employed in the chemical studies already published (*5, 6*). This series provided specimens up to and including the fifty-seventh day. Mice treated longer with the carcinogen were supplied by Doctors Cramer, Simpson, and Suntzeff. Some of these mice were of other closely inbred strains (table 1).

All mice were killed with chloroform, and except for Nos. 55 and 56, the tissues of all mice were excised within 1 hour of noon. This was done for the sake of uniformity and because there is a daily rhythm in frequency of epidermal mitoses which is at a maximum in mice at about this time (*7*).

In all cases squash preparations [2] were made and stained with acetocarmine (*4*). This method was used so that any doubling of chromosomes could be directly compared with the doubling in size previously reported in such preparations of other cancers and because whole mitoses are thereby displayed for examination. In addition, paraffin sections of tissues routinely fixed in Bouin's and Helly's fluids were stained, respectively, with haematoxylin and eosin, and with carbolpyronine and methyl green. These stained sections gave helpful information concerning the histologic features of the tissues in which mitotic figures were examined in squash preparations.

Correspondence between this series, used for chromosome studies, and the other series of similarly treated mice, previously investigated in Barnard Hospital, was checked by histologic study and found to be close.

On the chemical side, Dr. C. Carruthers has very kindly made determinations of iron and calcium at 10 and 29 days, using 10 mice for each of the 4 determinations. The results are given in table 2, with those already found in the standard Barnard Hospital series. These results are calculated on a weight basis, instead of as a ratio, because it did not seem necessary in this series to determine nucleoprotein phosphorus as a basis of reference. The similarity in trends (marked reduction in iron and proportionally slight reduction in calcium) is sufficient to justify the assumption that the chromosome modifications present in our series probably occurred also in the standard series, so that integration of observations is a safe procedure if the observations themselves are valid.

To obtain quantitative data on chromosome modifications involves careful study, with oil immersion lens and mechanical stage, of many squash preparations. The reason is that in stages of methylcholanthrene hyperplasia the average frequency of mitoses never exceeds 230 per 15,000 nuclei (*7*), and only

[1] Aided by grants from the National Cancer Institute and an anonymous donor.

[2] Figures 2-5 are photographs of these preparations made by W. G. Elle at a magnification of 1250.

TABLE 1.—*Frequency of enlarged chromosomes*

Mouse No.	Treatments			Metaphases examined	Metaphases with chromosomes		
	Chemical	Number	Days		Doubled	Quadrupled	Octupled
				Number	*Percent*	*Percent*	*Percent*
11	None		0	100	0		
12	do		0	100	0		
58	Benzene	1	1	50	0		
59	do	1	1	50	0		
13	do	1	3	150	0		
14	do	3	10	100	0		
15	do	3	10	240	0		
16	do	3	10	50	0		
54	Methylcholanthrene	1	½	50	0		
55	do	1	½	50	0		
56	do	1	1	50	0		
57	do	1	1	30	0		
49	do	1	2	50	2?		
50	do	1	2	50	2		
7	do	1	3	50	12		
8	do	1	3	50	18	2	
9	do	1	3	100	10		
17	do	1	10	100	7		
18	do	1	10	60	10		
19	do	1	10	60	0		
21	do	2	10	50	10		
22	do	2	10	100	12		
23	do	2	10	50	4		
24	do	2	10	100	3		
26	do	3	10	100	0		
27	do	3	10	100	4		
28	do	3	10	50	8		
29	do	3	10	50	0		
45	do	3	10	50	16		
46	do	3	10	50	4		
47	do	3	10	50	26	2	
48	do	3	10	50	4		
30	do	6	20	50	4	2	
31	do	6	20	50	10	2	
32	do	6	20	50	10		
33	do	6	20	50	4		
34	do	13	29	50	4		
35	do	13	29	50	2?		
36	do	13	29	50	18	2	
37	do	13	29	50	6		
38	do	25	57	100	5		
39	do	25	57	100	5		
40	do	25	57	50	2		
41	do	25	57	150	0		
2 [1]	do	6	72	50	38	4	
3 [1]	do	double 6	72	50	26		
42	do	double 33	86	30	20		
43 [2]	do	36	94	50	6		
4 [1]	do	5	108	50	20		
6 [1]	do	5	109	25	4?		
IN CARCINOMAS							
42	Methylcholanthrene.	33	86	50	76	4	
43 [1]	do	36	94	50	52	20	
4 [2]	do	5	108	50	70		
5 [2]	do	5	108	50	54		
5 [2]	do	5	108	50	60	24	2
6 [2]	do	5	109	10	40	50	
44 [1]	do	36	169	50	76	4	2
IN PAPILLOMAS							
41	Methylcholanthrene	25	57	130	1		
3 [2]	do	6	72	50	2		
1 [2]	[3]	double		60	0		

[1] Swiss mice. [2] CBA mice. [3] Spontaneous.

TABLE 2.—*Chemical analysis of mouse epidermis*

Number of paintings	Determinations	Metal	Metal per 100 mg. epidermis	
			This series	Standard series [1]
	Days		*Micrograms*	*Micrograms*
3	10	Fe	3.6	2.4
		Ca	28.0	19.0
13	29	Fe	2.8	1.5
		Ca	22.0	16.0

[1] Carruthers and Suntzeff (*5*, *6*).

the metaphases yield reliable information on chromosome enlargement.

Fifty metaphases seem a small number to examine in most specimens. However, better knowledge of the trend of change is reached by such study of several specimens at each time interval than by further concentration on a single specimen, for, by averaging, the individual variability shown in table 1 is to some extent ironed out. The statistical significance of the changes in frequency of enlarged chromosomes is considered later.

That the metaphase chromosomes in this material tend to occur in definite multiples of the original volume was discovered by measurements on 134 camera lucida drawings. The measurements showed a polymodal distribution of average chromosome volumes with peaks of frequency at $0.5\mu^3$ to $0.6\mu^3$, $1.0\mu^3$ to $1.2\mu^3$, and about $2.1\mu^3$. The three groups of chromosome volumes thus distinguished had mean values of approximately $0.5\mu^3$, $1.1\mu^3$, and $2.1\mu^3$. These average volumes for monochromosomes, diplochromosomes, and tetraplochromosomes, respectively, agree well with those determined in other tissues (*4*). The fact that modal frequencies of chromosome volume occur at approximate doublings of one another indicates that the enlarged chromosomes are really composed of more chromosome strands than normally and are not merely swollen, perhaps by hydration. This is also suggested by the fact reported earlier (*4*) that the large resting (nondividing) nuclei carrying enlarged chromosomes in cancers have multiples of the basic number of plasmosomes, which are products of chromosome-carried nucleolar organizers.

OBSERVATIONS

Figure 1 shows the percentages of enlarged chromosomes (doubled, quadrupled, and octupled) plotted against the time of treatment in days. This graph presents the tendency to change in frequency better than table 1, because for all the stages up to and including 72 days the figures are averages of specimens from several mice so that indi-

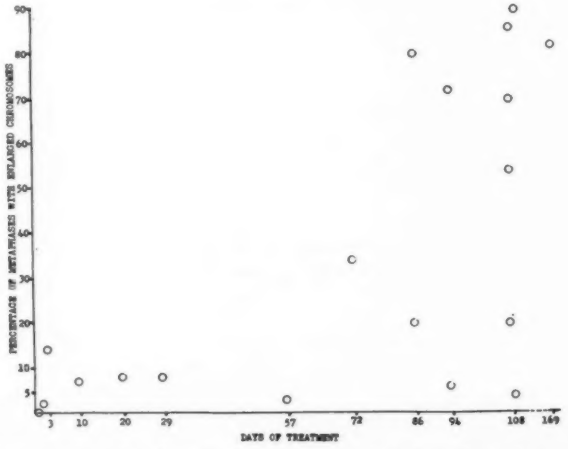

FIGURE 1.—Percentages of enlarged chromosomes plotted against time of treatment

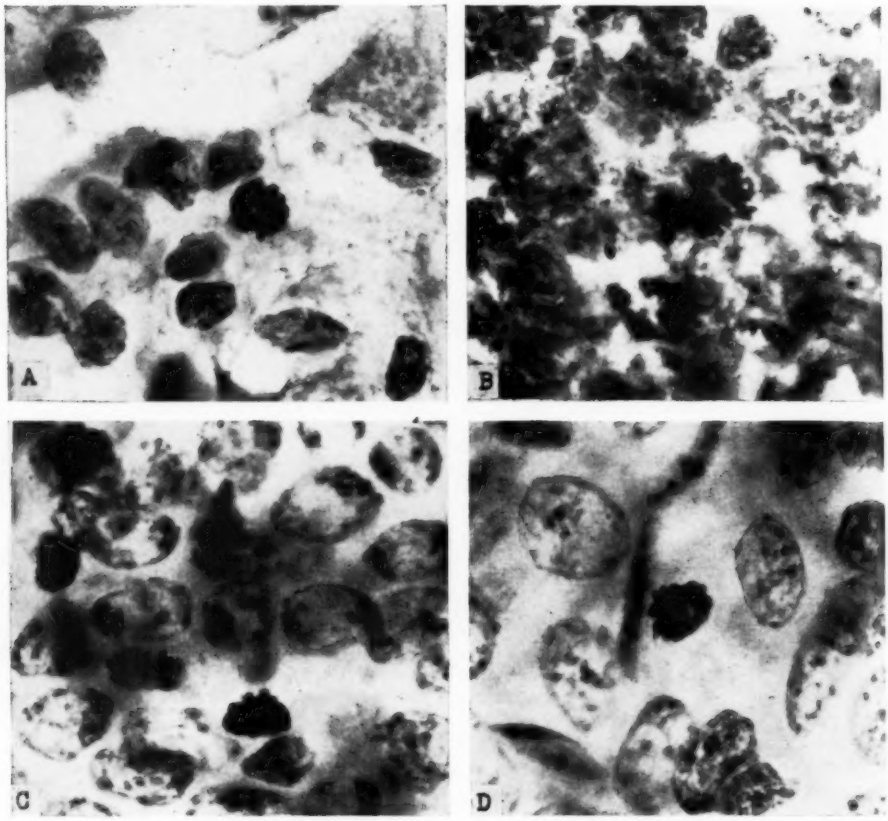

FIGURE 2.—*A* and *B*, Control, untreated epidermis. Normal chromosomes; *C* and *D*, Control. Three days after one application of benzene. Apparently normal chromosomes.

vidual variability is to some extent suppressed. But the later percentages are of single hyperplastic epidermises, or of single cancers. These fall in two groups, the first all below and the second all above 50 percent.

The purpose of this study is not only to ascertain the frequency of enlarged chromosomes but also to integrate this with the other alterations that occur. It is logical, therefore, to describe the chromosomal modifications at each stage and immediately thereafter to note what has been discovered in the standard series concerning the other concurrent changes.

In normal untreated epidermis 200 metaphases were examined, and no doubled chromosomes were found (table 1). In epidermises treated only with benzene, no doubled chromosomes were observed in 640 metaphases. Figure 2, *A-B*, and *C-D*, illustrate such metaphases in normal and benzene-treated epidermises, respectively.

One-half and one day after a single painting with methylcholanthrene in benzene, no doubled chromosomes were seen. Since the number of mitoses studied was not great, it cannot be said that metaphases with enlarged chromosomes were altogether absent.

At 2 days 1 mouse showed 1 metaphase in 50 examined in which the chromosomes seemed large and may have been

doubled; in another mouse 1 metaphase among 50 exhibited a definite doubling in chromosome volume. This 2-percent difference in frequency between the metaphases at 1 day and those at 2 days is not statistically significant, but the positive observation of an instance of doubling marks, to the best of our knowledge, the earliest appearance of this kind of nuclear modification in our series.

At this time in the other series interesting changes were observed. The frequency of mitosis increased from the normal of 17 per 15,000 nuclei to 26.3 per 15,000 (*7*), and the lipid/protein nitrogen ratio of the epidermis decreased from 5.25 to 4.28 (*8*).

At 3 days the frequency of enlarged chromosomes increased materially. (See sharp rise in figure 1.) The specimens from all three mice contained over 10 percent, one as many as 20 percent, including 2 percent of quadrupled chromosomes, making altogether a 13-percent frequency of metaphases with enlarged chromosomes.

This increase is of statistical significance. Table 3 shows that the probability that two samples from the same population should differ to this or a greater extent because of random fluctuation is of the order of two chances in a thousand (0.0019). It is considered that a probability of three chances or less in a thousand indicates a significant difference in the samples (*9, p. 122*). It shows, in other words, that the two samples were probably drawn from different populations. Estimations of statistical significance of the difference in percentages of metaphases with enlarged chromosomes between various samples were computed according to a method involving use of the standard deviation of the difference in two percentages (*9*). This standard deviation was calculated as the square root of the product of the total percentage of enlarged-chromosome metaphases and the total percentage of normal metaphases times the sum of the reciprocals of the two totals of all metaphases in each of the two samples. The difference in the two percentages was then divided by the standard deviation. If the resulting quotient was three or more, the probability that the difference between the two samples arose from random variation in sampling was about 0.003 or less. A difference giving rise to such a low probability was considered significant. Table 3 shows the significance of a number of variations in the

TABLE 3.—*Significance of variations in frequency of metaphases with enlarged chromosomes*

Group	Metaphases		Difference (D)	S. D. D	D / S. D. D	Probability [1]	Significance
	Total	With enlarged chromosomes					
	Number	*Percent*	*Percent*	*Percent*			
Control	840	0.0	6.8	0.8	8.5	0.0000	++
½–57 days	2,400	6.8					
2 days	100	2.0	11.0	3.6	3.1	.0019	+
3 days	200	13.0					
3 days	200	13.0	6.2	2.1	3.0	.0027	+
10 days	1,020	6.8					
10 days	1,020	6.8	1.2	2.0	.6	.5	−
20 days	200	8.0					
29 days	200	8.0	5.2	1.8	2.9	.0037	?
57 days	400	2.8					
3–57 days	2,020	6.8	14.4	1.8	8.0	.0000	++
72–109 days	255	21.2					
Treated epidermis	2,655	7.3	67.2	2.1	32.0	.0000	+++
Carcinomas	310	74.5					

[1] Where one is taken as certainty, that a difference as great as or greater than the given one will result from an error in sampling.

frequency of metaphases with enlarged chromosomes. Briefly stated, the differences between the following pairs are significant: 2 and 3 days, 3 and 10 days, 3—57 and 72—109 days, as well as between treated epidermis and cancers. Needless to say, the difference between the controls and the treated specimens is significant.

Not only was the chromosome enlargement at 3 days similar to that observed in cancer cells (*4*), but the aberration in chromosome numbers, often associated with malignancy, was also pronounced in certain cells at this early stage in carcinogenesis. Thus, figure 3, *A*, is of a prometaphase of 69 (instead of the diploid number of 40) diplochromosomes averaging $1.4\mu^3$. Figure 3, *B*, represents a metaphase of about 88 polytene chromosomes with an average volume of $3.0\mu^3$. Evidently the number of chromosomes has been doubled, and the amount of material in each chromosome has been increased perhaps 4 or 8 times, giving a total increase of 8 to 16 times in 3 days. In figure 3, *C*, a tetraploid metaphase with diplochromosomes is shown, with a fourfold increase in chromosomal material above normal. In figure 3, *D*, the

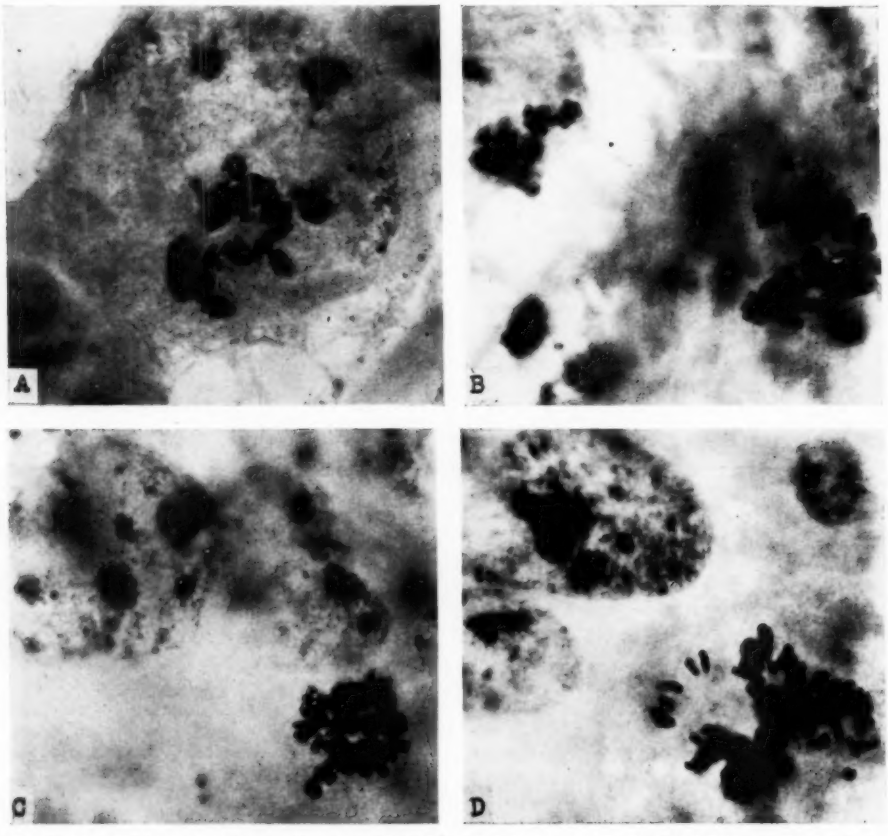

FIGURE 3.—Epidermis 3 days after 1 application of 0.6 percent methylcholanthrene in benzene. Marked chromosome changes. *A*, Doubled chromosomes (69) averaging $1.4\mu^3$; *B*, Enlarged chromosomes (88) averaging $3.0\mu^3$; *C*, Tetraploid number of diplochromosomes; *D*, Diplochromosomes (129) averaging $1.1\mu^3$. From the large dimensions of the nondividing nuclei the cells are probably spinous.

diplochromosomes number 129, and have an average volume of 1.1μ^3, a sevenfold increase. Such increased amounts of chromosomal material are inevitably reflected in the presence of large nondividing nuclei, as is indicated in figure 3, *A–D*. In comparing them with those in the normal or benzene controls (fig. 2, *A–D*), it is important to remember that the photomicrographs are not of sections, but in all cases of whole nuclei which have been flattened to about the same degree in making the acetocarmine squash preparations.

However, these variations in chromosome numbers were not very frequent in the metaphases which were drawn. Fifty-eight metaphases with chromosomes of normal size, taken in the course of epidermal carcinogenesis, yielded an average of about 43 chromosomes from numbers ranging from 27+ to 59. The extent of the aberration in numbers in the enlarged chromosome metaphases in carcinogenesis is not much greater, since 51 metaphases in the diploid neighborhood also had an average chromosome number of 43, with a range from 24 to 59. The standard deviation of chromosome numbers for the normal-chromosome metaphases was 7.14, and that of the diplochromosomal metaphases was 8.27. Some of the variations in number may have been more apparent than real. Seven enlarged-chromosome metaphases with chromosome numbers from 64 to 88 had an average of 75 chromosomes each. This is not too far from the ideal tetraploid number, 80. No polypoidy was found in small chromosome metaphases.

At this third day, when the chromosome modifications reached an early peak of frequency, the lipid/protein nitrogen ratio was further decreased to 3.72 in the other series (*8*), and, thanks to Dr. Carruthers, analyses of our series showed that the iron content per 100 mg. of epidermis was 7.8 micrograms, which is substantially normal, since his figure for normal epidermis is 6.4 micrograms.

At 10 days, more data are brought together concerning both the frequency of metaphases with enlarged chromosomes and the probable condition of the epidermis in which they occurred.

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The appearance of the doubled chromosomes was the same as that illustrated at 3 days, and again there was 1 instance of quadrupled chromosomes in 50 metaphases examined in the hyperplastic epidermis of 1 mouse (No. 47), a frequency in this case of 2 percent. When all 15 animals treated 10 days are considered, the frequency of enlarged chromosomes in the 1,020 metaphases examined is 6.8 percent; this decrease from the initial peak of 13 percent at 3 days was certainly not expected. This is perhaps a statistically significant change, for there are about 3 chances in 1,000 that a difference as great as or greater than this would result from a sampling error, as shown in table 3. The difference in the frequencies of enlarged chromosome metaphases after 1, 2, or 3 paintings at 10 days is, however, insignificant; after 1 painting the frequency was 5.7 percent; after 2, 7.3 percent; and after 3, 6.8 percent.

Mitotic frequency at this time was probably further increased, although this was not determined at 10 days because at 9 days it had risen to 78 per 15,000 nuclei (*7*). The lipid/protein nitrogen ratio at 10 days was 2.53, which was a distinct decline from that at 3 days, and a reduction from the normal of about 50 percent (*8*). The iron/nucleoprotein phosphorus ratio decreased from a normal of 52 to 27; but the ascorbic acid/nucleoprotein phosphorus ratio was not significantly changed, since the decrease was only from 0.193 to 0.167 (*5*). At this time it was observed (*6*) that the sodium/nucleoprotein phosphorus ratio increased only from 1.17 to 1.24, while the calcium/nucleoprotein phosphorus ratio dropped from 3.60 to 1.58. It is likely that decreases in iron and calcium, amounting to about 50 percent, commenced some time before 10 days. Two and even three applications of methylcholanthrene during the first 10 days did not appear to change the values greatly as compared with those obtained after a single application, even though the methylcholanthrene-induced fluorescence in the skin disappeared 6 to 10 days after a single application of carcinogen (*10*).

At 20 days, when the mice had been

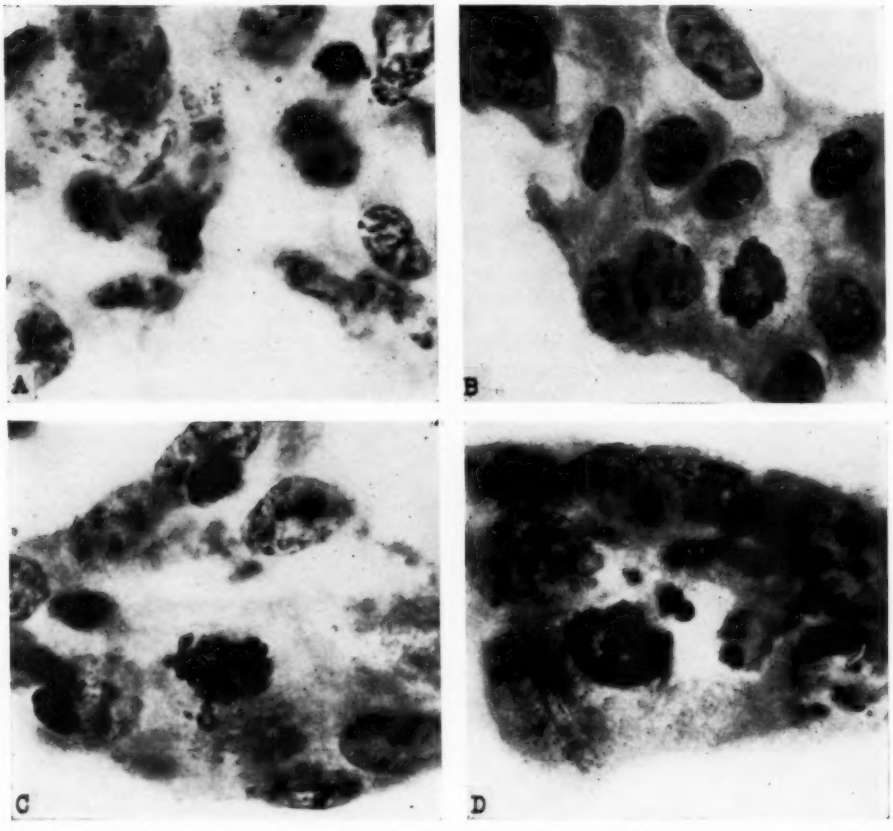

FIGURE 4.—Epidermis at 20 days, after six paintings with methylcholanthrene. *A*, Apparently normal chromosomes; *B*, Diplochromosomes probably diploid in number; *C*, Diplochromosomes (57) averaging 1.1μ^3; *D*, Quadrupled chromosomes (41) averaging 2.2μ^3.

given 6 treatments with carcinogen, the frequency of metaphases made up of enlarged chromosomes was only 8 percent, a statistically insignificant change from the 6.8 percent observed at 10 days. Metaphases typical of those appearing at 20 days are illustrated in figure 4, *A–D*. Figure 4, *A*, shows 1 made up of what look like normal chromosomes, which is the usual finding until cancer arises. Diplochromosomal metaphases are seen in figure 4, *B* and *C*, while figure 4, *D*, shows a metaphase containing about 41 chromosomes averaging 2.2μ^3. Although the epidermal nuclei in figure 4, *A–D*, are smaller than those in figure 3, *A–D*, they do not have the watery appearance of those treated with benzene alone, and many of them are larger than those in normal epidermis.

The plateau in frequency of enlarged chromosomes (fig. 1), of which 20 days is the mid-point, has its counterpart in an equilibrium of some of the other properties measured. Chemical analyses, as detailed by Carruthers and Suntzeff (*11*), show that sodium, calcium, potassium, iron, and ascorbic acid are maintained at a fairly constant level up to 60 days. The nucleocytoplasmic ratio changes very little although there is an

increase in volume of both nucleus and cytoplasm (*12*). Visual examination of sections reveals a slight increase in the displaceability of nucleoli and basophilic chromatin under the influence of ultracentrifugal force, but this alteration has not been quantitatively measured (*13*). During this period of comparative equilibrium the hyperplasia increases, but the cells are fairly uniform in appearance at the several epidermal levels.

Yet it is likely that at 20 days the frequency of mitoses had increased, for at 23 days in the other series 149.7 were observed per 15,000 nuclei (*7*). At 24 days (9 paintings), the content of thymonucleic acid per nucleus had advanced until it was intermediate between that of normal skin and carcinoma (*14*).

At 29 days (13 treatments), the enlarged chromosomes still exhibited a frequency of 8 percent. At 30 days, the frequency of mitoses had started to decline from the minor peak at 23 days (*7*). Variation in the other properties was of moment only in the iron content, which dropped from about 2.5 micrograms per 100 mg. of tissue near 20 days to 1.5 micrograms at 30 days (*5*).

At 57 days (25 treatments), the frequency of diplochromosomes was at the low value of 2.8 percent of the 400 metaphases examined, but the frequency was not determined at any interval be-

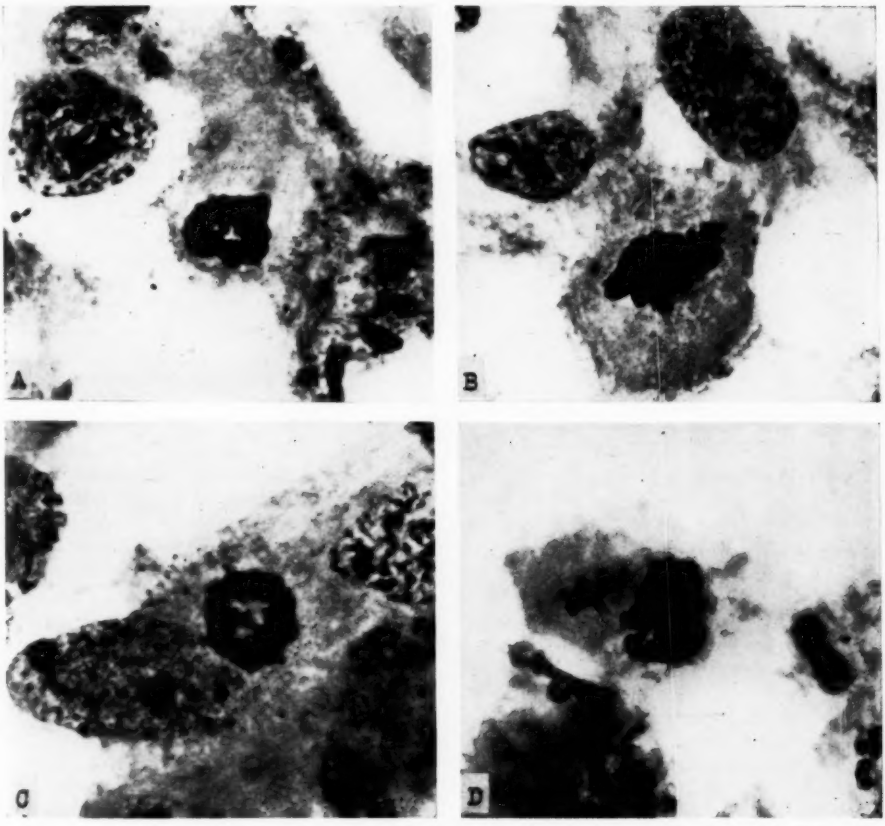

FIGURE 5.—Carcinomas. *A*, *B*, and *C* probably contain diplochromosomes. *D*, Quadrupled chromosomes (56) averaging 2.3μ^3.

tween 29 and 57 days in this series. (However, 3 Swiss mice showed a frequency of 6.7 percent for 150 metaphases at 56 days after a double painting.) Consequently, it is not safe to join these 2 points (or any others) by a straight line in the chart. Although the drop to 2.8 percent is of doubtful significance (see table 3), yet it seems to be parallel to the decided drop in mitotic frequency, which reached a low of 72.7 mitoses per 15,000 nuclei at 65 days (*7*).

At 72, 86, 94, 108, and 109 days, the treated epidermises displayed an average of 20 percent enlarged chromosomes. One animal of the six even had the near-cancerous value of 38 percent diplochromosomes and 4 percent quadrupled chromosomes in 50 metaphases examined. Parallel to this increasing frequency, there was also an increase in the mitotic frequency to 162 per 15,000 nuclei at 72 days. After a slight fall the number of mitoses attained 225 per 15,000 nuclei at 93 days (*7*).

In 7 carcinomas the frequency of metaphases containing enlarged chromosomes was again increased, but the character of the change was similar. Figure 5, *A-D*, illustrates metaphases containing varying numbers of diplochromosomes; figure 5, *D*, shows a metaphase of 56 tetraplochromosomes. In it are 6 lagging chromosomes to the left of the main group. In all of them, the frequency of enlarged chromosomes was greater than in any of the hyperplastic epidermises examined. But there was considerable variation between individuals. (See table 1 and figure 1.) Even in the same mouse (No. 5), 2 cancers showed quite different frequencies of doubling: one had very few nuclei larger than double the volume of normal epidermal nuclei and contained 54 percent diplochromosomes; the other had many huge nuclei and showed diplochromosomes in 60 percent of its dividing cells, tetraplochromosomes in 24 percent, and octoplochromosomes in 2 percent.

To link these observations with data obtained relative to the standard series is difficult. While the greatest increase in frequency of enlarged chromosomes took place in the transition from hyperplasia to malignancy, there was no appreciable alteration in the ascorbic acid/nucleoprotein phosphorus ratio. We have to wait for other information on the chemical constitution of the cancers until a method is devised for correcting the analyses so that they relate to the epithelial part of the tumors and are not vitiated by the presence of blood and other components absent in the avascular epidermises. But the large differences in cellular and nuclear volume reported in the standard series (*12*) may not be related to differences in chromosome values.

In the three papillomas examined, there was a very low frequency of enlarged chromosomes, about 1 percent. This is in excellent agreement with Stowell's observation (*14*) of very little or no increase in thymonucleic acid per nucleus in papillomas as compared with normal epidermis.

DISCUSSION

The appearance of doubled chromosomes as early as 48 hours after a single application of methylcholanthrene was unexpected because at this time the association of carcinogen with the cells could only have been slight and of short duration. However, Creech (*15*) observed precocious splitting of chromosomes in tissue cultures treated with 1, 2, 5, 6-dibenzanthracene choleic acid at 2 days.

Though enlarged chromosomes have been found to be of frequent occurrence in cancers (2, 3, 4) (in our methylcholanthrene carcinomas their frequency was over 50 percent); it does not follow that this modification is necessarily indicative of malignancy because it has also been reported in noncancerous cells (*4*, *16*). But the enlargement does bear witness to a profound nuclear alteration, which at 3 days was detected in 13 percent of the metaphases examined. If the theory is correct that the doubling and quadrupling of chromosome size is occasioned by endomitosis (internal mitosis without nuclear or cell division) and that the resulting chromosomes are many-stranded, or polytene, there is every reason to think that the nucleoprotein content remains the same per

unit of volume, as would indeed appear to be the case from their tinctorial properties. It would appear, therefore, that in the cells that display polytene chromosomes there is an unusually high concentration of thymonucleoprotein per cell which might be explained on the basis either of a hypothetical increase in rate of synthesis or of synthesis over a long period of time. Evidence of increased synthesis is given not only by the increase in frequency of mitosis in the companion series (*7*) but also by the observation of a slight increase in thymonucleic acid content per nucleus after nine paintings over 24 days (*14*).

Also interesting were extensive aberrations in chromosome number at 3 days. It almost looks as if Koller's three long steps to cancer (*17*) are taken in this brief interval, as far as the chromosomes are concerned. However, extreme changes in chromosome number occurred before much increase in heterochromatin was noted, thus reversing some of his stages. Blum (*18*) discussed the possibility that tumor cells may be present long before a cancer can be histologically demonstrated.

We do not, however, suggest that these remarkable cells with doubled, quadrupled, and otherwise abnormal chromosomes are necessarily cancerous and are locked up in the epidermis so that they cannot invade the dermis. Since the classic work of Boveri (*19*), the possibility must be entertained that abnormal chromosome complexes may have a lethal influence on the cells that bear them. Obviously, it is impossible in this carcinogenic series to determine how long individual cells modified in this way endure, and the fate of their offspring, if any.

For some reason, difficult to explain, the increase in frequency of such cells from 2 to 3 days was not continued. Instead of finding a further increase at 10 days, parallel to the great increase in frequency of mitosis, the percentage of metaphases with enlarged chromosomes actually decreased. The decrease, however, was not progressive, for the frequency showed no significant alteration at 29 and 57 days from that determined at 10 days. As far as our observations go, this equilibrium in the frequency of enlarged chromosomes corresponds roughly in time to the new chemical equilibrium in the reacting epidermis characterized by a decrease of about 50 percent in iron and calcium while the amounts of ascorbic acid, magnesium, and potassium remain about the same. Both changes in chromosomes and in chemical composition are to a remarkable degree independent of frequency of treatment with methylcholanthrene. To determine which is primary and which secondary is beyond us. The change in chemical composition may be occasioned by a multiplicity of forces set in operation by the carcinogen and may prove to be far more complex than the data now available demonstrate.

But during this precancerous equilibrium, other properties of the tissue, in addition to frequency of mitosis, do change. Thus, there is a progressive increase in cell and nuclear volume although the nucléocytoplasmic ratio remains about the same. This may be brought about by an increase in water content, which itself might explain the increase in displaceability of nuclear chromatin and nucleoli subjected to ultracentrifugal force. Probably a kind of maturation proceeds which later progresses much faster in certain foci than in others with the result that in tissue not yet diagnosable as cancerous there appears great diversity in frequency of chromosomal changes and in some places these changes approach in frequency those found in cancers. Perhaps the chromosomal changes will serve as clues to chemical alterations in such foci which are dampened out by lack of change in the remainder of the tissue, since the whole of it is included in the analyses.

Obviously, this integrative investigation of carcinogenesis at Barnard Hospital has just been commenced. There remain many more questions than can be answered. Among them is the influence of alterations in chemical environment on the nucleus.

SUMMARY

The chromosomes were studied, by means of the acetocarmine technique, in mouse epidermis that had been treated with methylcholanthrene in benzene. Diplochromosomes, and even more greatly enlarged chromosomes were found from the second day on through carcinogenesis, while only normal chromosomes were present in normal and in benzene-painted control epidermises. Despite additional paintings, the frequency of enlarged chromosomes remained at about 8 percent through the first 2 months but increased later to values over 50 percent in the cancers.

The relation of these chromosomal changes to chemical and other changes in methylcholanthrene-induced epidermal carcinogenesis is discussed.

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STUDIES ON HEPATOMAS. I. SIZE AND SPACING OF MULTIPLE DOSES IN THE INDUCTION OF CARBON TETRACHLORIDE HEPATOMAS

By ALLEN B. ESCHENBRENNER, *passed assistant surgeon,* with the technical assistance of ELIZA MILLER, *National Cancer Institute, National Institute of Health, United States Public Health Service*

The induction of hepatomas in mice by repeated administration of carbon tetrachloride was first reported by Edwards (*1*). In 2 subsequent papers Edwards and Dalton (*2*) and Edwards, Heston, and Dalton (*3*) reported the induction of these tumors in 5 different strains of mice (A, C, C3H, L, and Y). They (*2*) recorded the histopathology of the hepatomas and of the livers following administration of this agent and presented some preliminary dose-response data. It was shown that although 3 large doses of carbon tetrachloride totaling 0.12 cc. produced no hepatomas, this quantity divided into 25 or 30 doses and given 2 to 3 days apart produced hepatomas in 41 of 58 mice. In addition, a high incidence of hepatomas was obtained both in animals that received 0.04 cc. of carbon tetrachloride per dose and in those that received 0.005 cc. per dose for a total of more than 23 doses given 2 to 3 days apart.

Under the conditions of these reported experiments some factors in addition to that of the total amount of the agent administered seemed to be influencing the induction of these tumors. It is conceivable that the duration of exposure to the agent as well as the interval between successive doses might also be significant factors. It is known that the time factor in exposure to a carcinogenic agent is important, but it has been studied largely under conditions of continuous exposure. Carbon tetrachloride, however, probably exists as such in the body for only a few hours, so that in this instance one must consider the possible effects of variations of the rate of intermittency of exposure as well as variations of total time of exposure to the agent. This report covers the first of a series of experiments designed to study the factors of time and dose in carcinogenesis by intermittent exposure. The size of individual doses and the interval in days between successive doses have been varied while the total number of doses and the time elapsing between the first dose and final examination of the animals for the presence of hepatomas have been kept constant. Since the total number of doses is constant, a variation in time between successive doses will also represent a variation in the total length of time during which these doses are administered.

EXPERIMENTAL PROCEDURE

The animals used were strain A mice procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and from colonies of this strain maintained at the National Cancer Institute. This strain has an incidence of spontaneous hepatomas of 1 percent or less (*2*). The mice were 2½ to 3 months of age at the beginning of the experiment. Male and female animals were segregated. They were fed Purina dog chow pellets and tap water ad libitum. Environmental temperatures were maintained at 78° F. The carbon tetrachloride was of chemically pure quality and was diluted with U. S. P. olive oil. Solutions were stored at 42° F. when not in use. The solutions of carbon tetrachloride in olive oil were administered by stomach tube.[1]

All animals received 30 doses of solutions of carbon tetrachloride in olive oil or of olive oil alone. Five dilutions of carbon tetrachloride were used: 32, 16, 8, 4, and 2-percent solutions. All animals received 0.005 cc. of solution per gram body weight containing 16×10^{-4}, 8×10^{-4}, 4×10^{-4}, 2×10^{-4}, or 1×10^{-4} cc., respectively, of carbon tetrachloride. Central necrosis of the liver lobule is produced by each of these doses. Control animals received 0.005 cc. of olive oil per gram body weight. Each of these dosage and control groups was further divided into 5 subgroups according to the interval between successive doses (1, 2, 3, 4, and 5 days) or to the total period of intermittent exposure to the agent (29, 58, 87,

[1] The stomach tube used in these experiments was devised by Morris and Thompson (*4*).

116, and 145 days). Equal numbers of male and female mice were used in each of the 25 experimental and the 5 control groups. Although the experimental procedure for a given animal was of only 5 months' duration, these experiments were carried out over a period of 13 months. However, at any given time approximately equal numbers of animals in all were not present, laparotomy was performed at monthly intervals thereafter to determine if any hepatomas eventually did appear in such animals. In either case the gross diagnosis of hepatoma was confirmed by microscopic examination. Animals that died prior to 150 days were examined both grossly and microscopically.

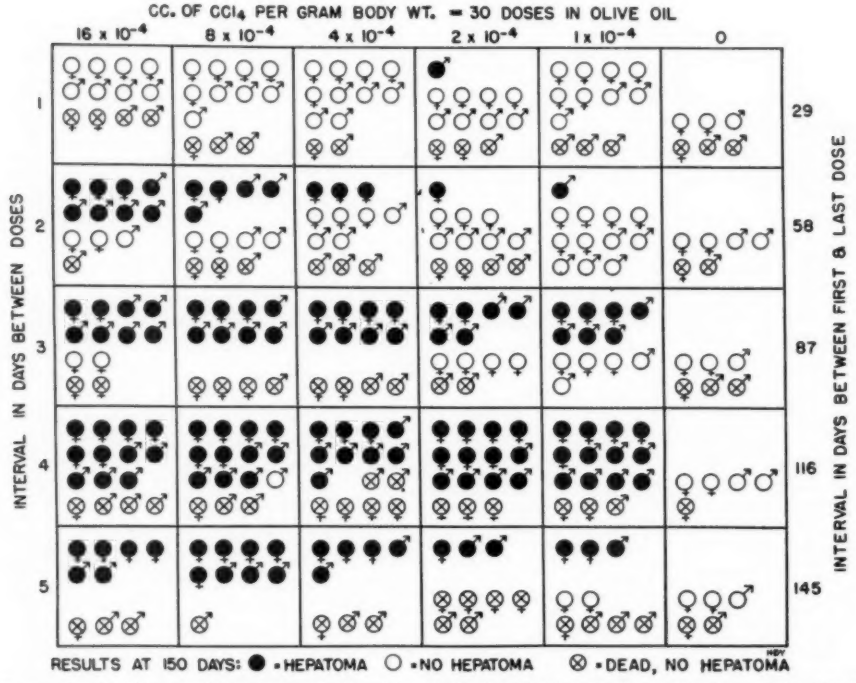

FIGURE 1.—Incidence of hepatomas induced in strain A mice by 30 doses of carbon tetrachloride in olive oil. Effect of variations in size of dose and in the interval between doses.

30 groups were under treatment so that any unknown variations of environment that might secondarily influence the production of these tumors are not likely to be reflected in the known variants. The general outline of the experiment is graphically represented in figure 1.

All animals were examined for the presence of hepatomas 150 days after the first dose. Some of them were killed at that time. Others were subjected to laparotomy at that time; and if hepatomas were present, the animals were used for other experiments. If hepatomas

RESULTS

The incidence of hepatomas in the various dosage groups is shown in figure 1. Animals that died prior to 150 days and were found to be tumor-free are recorded in figure 1 as "dead, no hepatoma." These deaths in a few instances resulted from mediastinitis following puncture of the esophagus by the feeding tube. In the majority of cases early deaths were due to chronic pneumonia. In the early stages this appeared as a mononuclear leukocytic infiltration of the submucosa

of the bronchioles; and as the disease progressed, more and more of the peribronchiolar alveoli were involved, often to the degree of producing complete consolidation of a lobe. Early deaths are seen from figure 1 to be of random distribution in all experimental and control groups.

Four mice that died prior to 150 days were found to have hepatomas. They are recorded as having hepatomas (fig. 1). Twelve mice in the 1-day interval groups and nine mice in the 2-day interval groups not showing hepatomas at 150 days were subjected to exploratory laparotomy at monthly intervals. Small nodules that proved to be hepatomas on microscopic examination appeared in four animals. These nodules were in mice that had received the doses 4×10^{-4} and 2×10^{-4} cc. at intervals of 1 day and in mice that had received 16×10^{-4} cc. at intervals of 2 days. The hepatomas were first observed between 210 and 240 days after the first dose of carbon tetrachloride.

In addition to the data presented in figure 1 showing only the incidence of hepatomas in the various dosage groups, observations on size and number of hepatomas are of interest. Hepatomas were not counted or measured, so the following is only a general impression of the observed differences which, however, were consistent and pronounced. In the lower dosage and shorter interval groups hepatomas were few in number and small. With increase of dosage and increase of interval between successive doses, there was a progressive increase in number of small hepatomas and in the size of hepatomas for a given animal.

For any given conditions of dosage of carbon tetrachloride used in this experiment the numerical incidence of hepatomas was observed to be approximately the same in both sexes.

Spontaneous hepatomas were found in three animals. Two of these were in experimental animals and one in a control animal. They were suspected of being spontaneous hepatomas grossly, by their yellowish color as contrasted with the pale pink color of induced hepatomas, and were distinguished from them microscopically by their architecture and by the structure of their mitochondria. The characteristics of the two types of hepatomas have been described in detail by Dalton and Edwards (*5*) and will not be repeated here.

DISCUSSION

The effects of variations of quantity and spacing of administration of carbon tetrachloride are limited in this experiment to conditions of a constant number of doses and of the incidence of hepatomas after a constant time from the beginning of exposure to the agent. Within these limits, the incidence of hepatoma increases with increase of total time during which a given amount of carbon tetrachloride is administered. On the other hand, with a given constant total time during which the agent is administered, the incidence of hepatomas increases with the total amount administered. Or, stated in another way, a given incidence of hepatomas is obtained with progressively less total amount of carbon tetrachloride as the duration of administration is increased.

The data do not reveal whether or not the variation in interval of time between successive doses is a significant factor in the observed variation of incidence. A point to bear in mind is that animals in the 5-day interval groups obviously acquired hepatomas before 30 doses were administered.

The results of laparotomies performed at monthly intervals on initially tumor free animals indicates that to a certain extent a variation of incidence relative to dose at a given time is a reflection also of the tumor development time. This is in agreement with studies on other carcinogenic agents of diverse nature.

The use of olive oil as a vehicle for diluting the carbon tetrachloride may impose a dietary factor in this experiment, but it would be constant for a given size of dose. It would be variable in any comparisons between different doses of carbon tetrachloride, although its effect could not conceivably be very large as compared with that of the carbon tetrachloride. The failure to obtain hepatomas with olive oil alone is in agreement with other reports (*1, 2, 3*).

Carbon tetrachloride produces necrosis of liver cells in the center of the lobules, and this necrosis is followed by rapid multiplication of those cells that escaped necrosis. Such a cycle when repeated many times might permit a progressive selection of the liver cells that have the greatest potentiality for division and for resistance to the lethal action of the agent. The question arises whether such a cyclic process plays any significant role in the genesis of these tumors. This may or may not be revealed by a comparison of the results of continuous and intermittent administration of a given amount of the agent over the same period of time. Regardless of whether or not a cyclic process is involved in the genesis of these tumors, a study of carcinogenesis by intermittent exposure to such an evanescent agent offers interesting possibilities for the study of the underlying mechanisms. The present data serve as a basis for such studies. Attention has recently been directed to such possibilities by Cramer and Stowell (*6*) in the instance of intermittent exposure of the skin to methylcholanthrene. Skin painting with this agent, however, introduces technical difficulties in accurate measurement of dose and in estimation of length of time the agent persists in the skin.

Spencer et al. (*7*, *8*) have recently observed a marked difference in the ability of small, free-living, single-celled species (bacteria) to adapt to an unfavorable environment when the exposure is discontinuous as compared with a continuous exposure.

SUMMARY

Thirty doses of carbon tetrachloride in olive oil were administered to mice by stomach tube. A variation in the numerical incidence of hepatomas at a given time was observed to be related both to the total amount administered and to the interval elapsing between successive doses. The influence of each of these two dependent variables is approximately of the same order so that the effects of variations of one of these must never be considered alone but always in terms of the other. The numerical incidence of hepatomas at a given time was found also to be related to the tumor-development time.

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A TRANSPLANTABLE OSTEOGENIC SARCOMA ORIGINATING IN A C3H MOUSE

By Morris K. Barrett, *formerly research fellow,* Albert J. Dalton, *cytologist,* Jesse E. Edwards, *formerly research fellow,* and Jesse P. Greenstein, *senior biochemist,* with the technical assistance of Virginia C. Briggs, *National Cancer Institute, National Institute of Health, United States Public Health Service*

The occurrence of bone tumors in mice has been reported by Murray (*1*), Bashford (*2*), Brunschwig and Harmon (*3*), and Pybus and Miller (*4, 5*). However, the histology and behavior during successive generations of transplants of the majority of those cases reported are dissimilar to the one described herein. Bashford's tumor strain 513 was apparently similar to the present one as a primary tumor, but the later history was different. Our tumor has been designated osteogenic sarcoma NCI No. 1 and is now being carried in routine transplant at the National Cancer Institute. At this writing, it is in the eighteenth generation.

HISTORY OF THE TUMOR

The original tumor was attached to the left thigh of a female C3H mouse of the Andervont line, and at the time of autopsy (Apr. 17, 1942) it was approximately 1.3 x 1 x 1 cm. in size. Transplants into the subcutaneous tissue of the lateral chest wall of three Andervont C3H female mice were made by the trocar technique, and the tumor grew in all three hosts. Subsequent transplants were made by the same technique into male and female mice of both the Andervont and Bar Harbor lines of the C3H strain. A slight increase in growth rate was noted in the first five generations, the tumor growing at an average rate of approximately 1.3 gm. per month. In the later generations, the growth rate has increased rapidly so that at the present time it is averaging more than 6 gm. per month. The number of successful transplants averaged 95 percent during the first five generations and in the later generations has been consistently 100 percent. No obvious sex difference has been noted, either in transplantability or in growth rate of the tumor, but the total number of animals in the series is at present too small for a final conclusion in this regard. From the seventh generation on, there has been no evidence of bone formation. Probably correlated with this change have been the increase in growth rate already mentioned and in addition the appearance of anaplastic cell types and greatly increased numbers of mitotic figures.

PATHOLOGY

GROSS EXAMINATION

The primary tumor was widely attached to the muscles of the thigh and to the deeper fascia but was not attached to the overlying skin. Attachment to the femur was suspected, but this suspicion was not borne out on dissection although the bone surface appeared to be etched. The tumor tissue was very hard and gritty on section. No metastases from the primary tumor were observed.

The transplants of the first six generations were all similar in appearance, presenting an irregularly lobulated surface covered by a fairly complete capsule. Hemorrhagic areas were regularly present, as well as small, centrally located areas of necrosis. Necrotic areas tended to have a more gritty character on section than did the more viable regions. Pulmonary metastases occurred regularly in hosts carrying transplants for 2 months or more. With the exception of one case in which metastases were discovered in the liver, metastases to organs other than the lungs were not noted in the first six generations. Beginning with the seventh generation, a change was noted in the gross appearance of the tumor. While it retained its irregularly lobulated surface, the capsule appeared much thinner and incomplete. The texture of the tumor varied from soft to firm but was never hard or gritty on section. At this time, a change in habits of metastasis was noted also. Regional lymph nodes as well as the lungs were found to be invaded by tumor cells. The tumor became highly invasive in character, frequently invading the

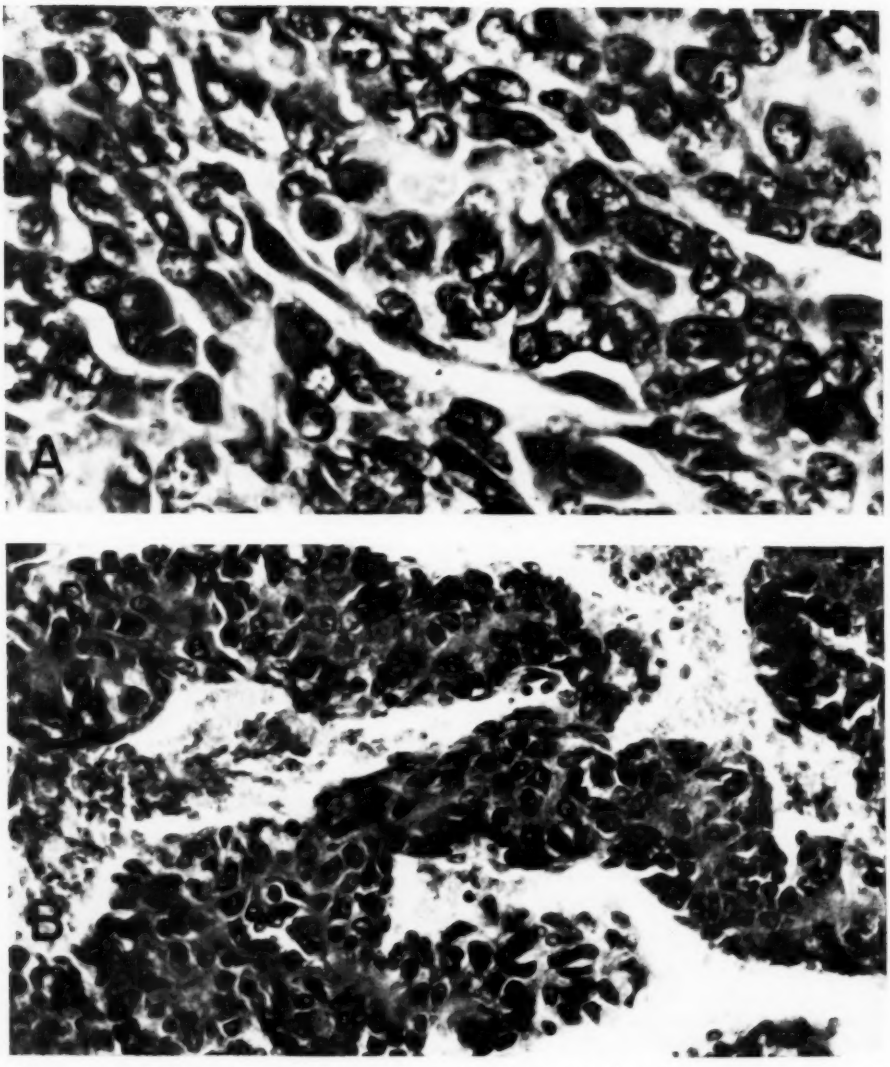

FIGURE 1.—*A*, Primary osteogenic sarcoma showing absence of the obvious cord arrangement characteristic of most areas of the tumor. Tumor cells (osteoblasts) and tumor giant cells are visible. × 490; *B*, Typical arrangement of the tumor cells in the form of fairly thick cords separated by wide sinusoids lined with endothelial cells derived from the host. × 150. Zenker-formol fixation and haematoxylin and eosin stain used in both *A* and *B*.

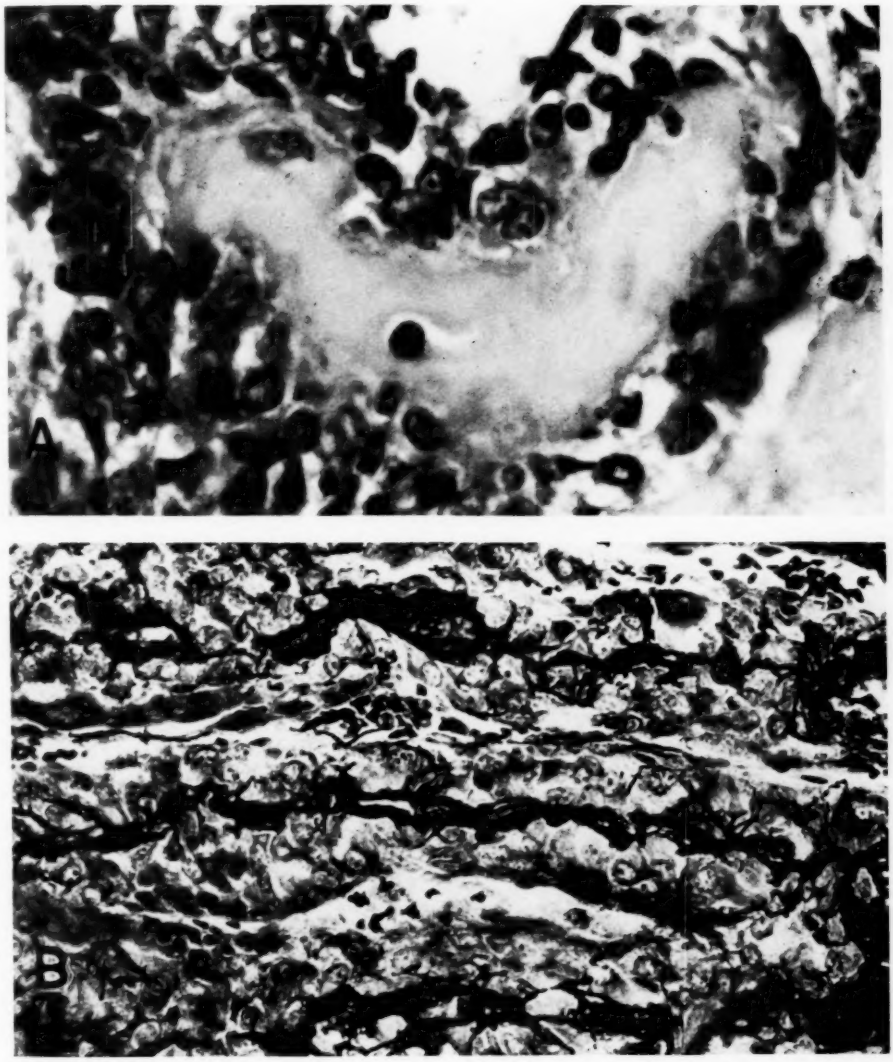

FIGURE 2.—*A*, Area of tumor in which osteoid tissue has been laid down. Zenker-formol fixation and haematoxylin and eosin stain. × 490; *B*, Arrangement of the reticulum characteristic of the tumor, with fine fibers occasionally extending between the individual cells. Zenker-formol fixation and Laidlaw's stain for reticulum. × 490.

peritoneal cavity and sometimes the pleural and pericardial cavities from its subcutaneous position. Peritoneal implants frequently resulted when the body cavity was invaded. In the later generations metastases appeared to occur much earlier and to grow more rapidly than in the earlier generations.

MICROSCOPIC EXAMINATION

Primary Tumor

The histologic type of the tumor is similar to that described by Pybus and Miller (*5*) under the classification "osteosarcoma with giant cells." The large majority of the cells were identified as osteoblasts, and interspersed between them were occasional giant cells similar in appearance to the osteoclasts seen in areas of normal growing bone except that the nuclei were frequently lobulated rather than spherical or ovoid in form (fig. 1, *A*). While there were a few foci in which cells lay haphazardly in a matrix of collagen (fig. 1, *A*), the usual arrangement was that in which the tumor cells took part in the formation of cords (fig. 1, *B*). The centers of the cords were composed of either collagenous or osteoid tissue (fig. 2, *A*), bordered by a single row, or occasionally a stratified layer of tumor cells, the nuclei of which tended to lie toward the outer margin of the cord. The tumor cells were either in direct contact with each other or were separated by filaments of reticulum which branched outward from the central collagenous or osteoid tissue of the cord (fig. 2, *B*).

The tumor cells were of moderate size, and cuboidal, columnar, or pyramidal in shape. The cytoplasm stained slightly basophilic, and the cell boundaries were clearly visible. No intracellular fibrils were demonstrable with Mallory's phosphotungstic acid-haematoxylin stain. The nuclei were spherical or ovoid, with prominent membranes, finely divided chromatin material, and indistinct, small nucleoli. Mitotic figures occurred in moderate numbers.

The medullary tissue of the femur involved in the primary growth showed replacement with trabeculae of bone, along the surfaces of which tumor cells were located. The cortex of the bone was permeated in several places by such cells, which in turn were continuous with the neoplastic tissue surrounding the bone. The tumor present in the soft tissue invaded the perineural lymphatics and skeletal muscle.

In none of the material of the primary tumor was cartilage identified.

Transplants

Structures seen in the primary tumor were in the main duplicated in the transplants of the first five generations. Cord-like areas alternating with endothelial-lined sinusoids (fig. 3, *A*) and more solid areas were again observed. Osteoid tissue and areas of osteogenesis (fig. 3, *B*) were prominent features of the early generations of transplants. Partial encapsulation, with invasion of the capsule by tumor cells, was present.

In two animals allowed to die 5 months after inoculation of tumor cells, numerous pulmonary metastases were found, most of which were located just beneath the visceral pleura (fig. 4, *A*). In one metastasis, there was extension through the pleura so that it lay partly in the pleural cavity . The portion of the metastatic growth lying under the pleura was spherical, while that lying in the pleural cavity resembled a flattened mushroom. True bone was present in greater amounts in that part of the metastasis lying in the pleural cavity (fig. 4, *B*). The structure of the secondary tumors was similar to that of the primary tumor and the transplant from which they were derived.

In the third and fourth generations, softer portions of the transplants were found, which on histologic examination appeared to be typical fibrosarcomas. However on transplantation of these soft portions, osteosarcoma reappeared in the next generation.

Beginning with the seventh generation and continuing to the present, a general change in structure has been noted. All these later generations have failed to show the presence of bone or osteoid tissue. The tumors have been highly cellular, exhibiting two main types of tissue. One type was represented by spindle-shaped cells occurring in strands. These cells had elongated nuclei with evenly distributed chromatin and with nucleoli which, when visible, were small

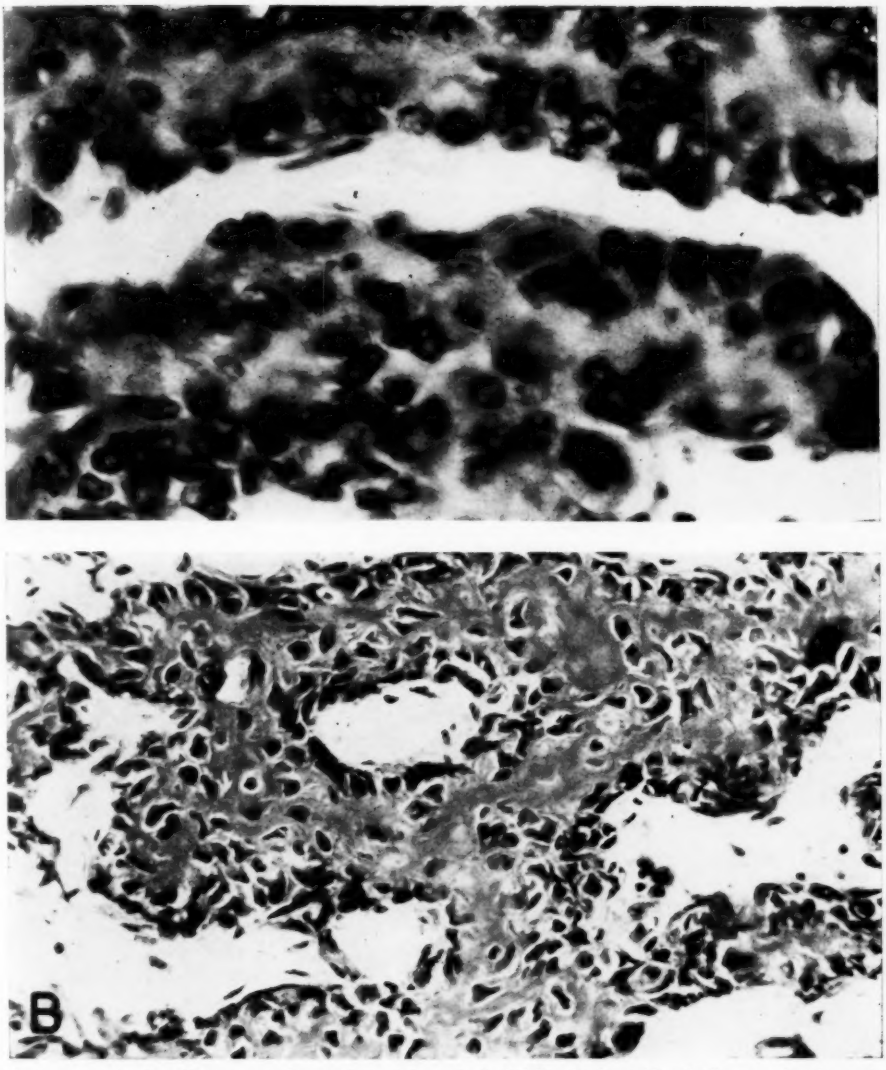

FIGURE 3.—*A*, Cordlike areas alternating with endothelial-lined sinusoids similar to the form in the primary tumor. × 490; *B*, Area of true bone formation. × 235. Both *A* and *B* from material from first-generation transplants fixed in Zenker-formol and stained with haematoxylin and eosin.

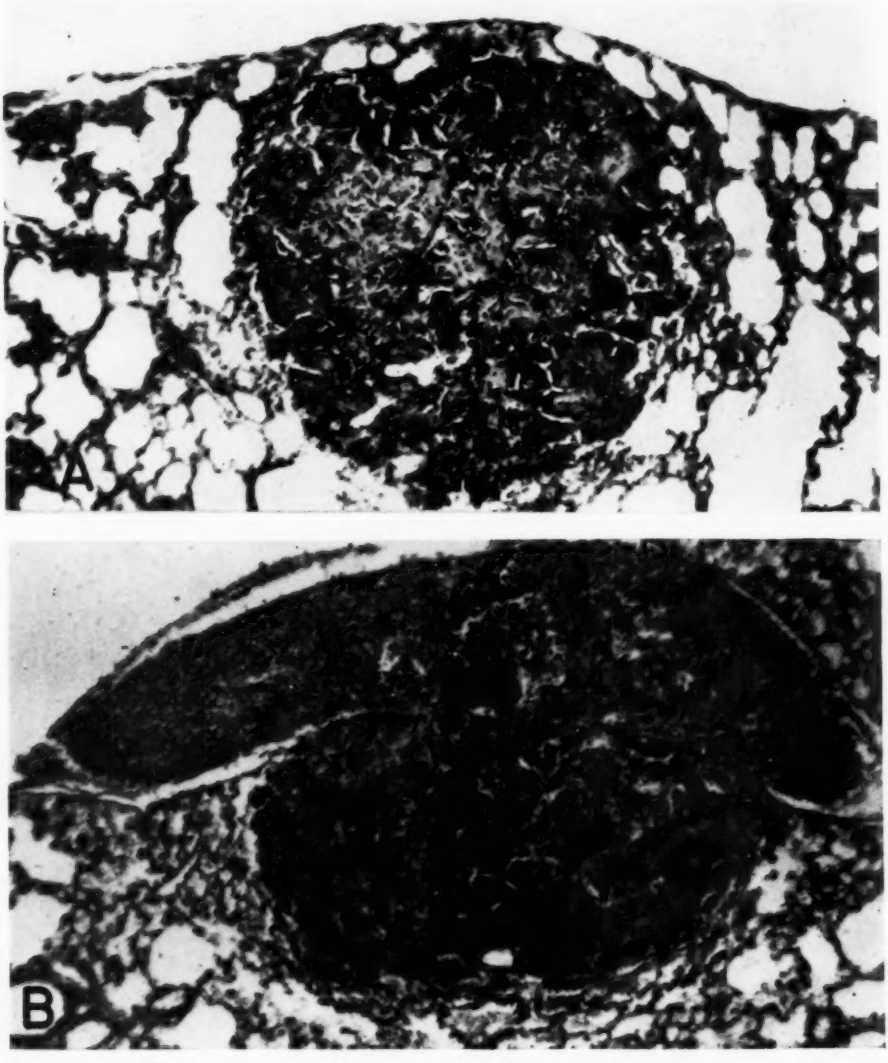

FIGURE 4.—*A*, Typical structure and form of the pulmonary metastases present in the early generations of transplants. *B*, Extension of a pulmonary metastasis through the visceral pleura. Both *A* and *B* from first-generation transplants, tissue fixed in Zenker-formol and stained with haematoxylin and eosin. × 20.

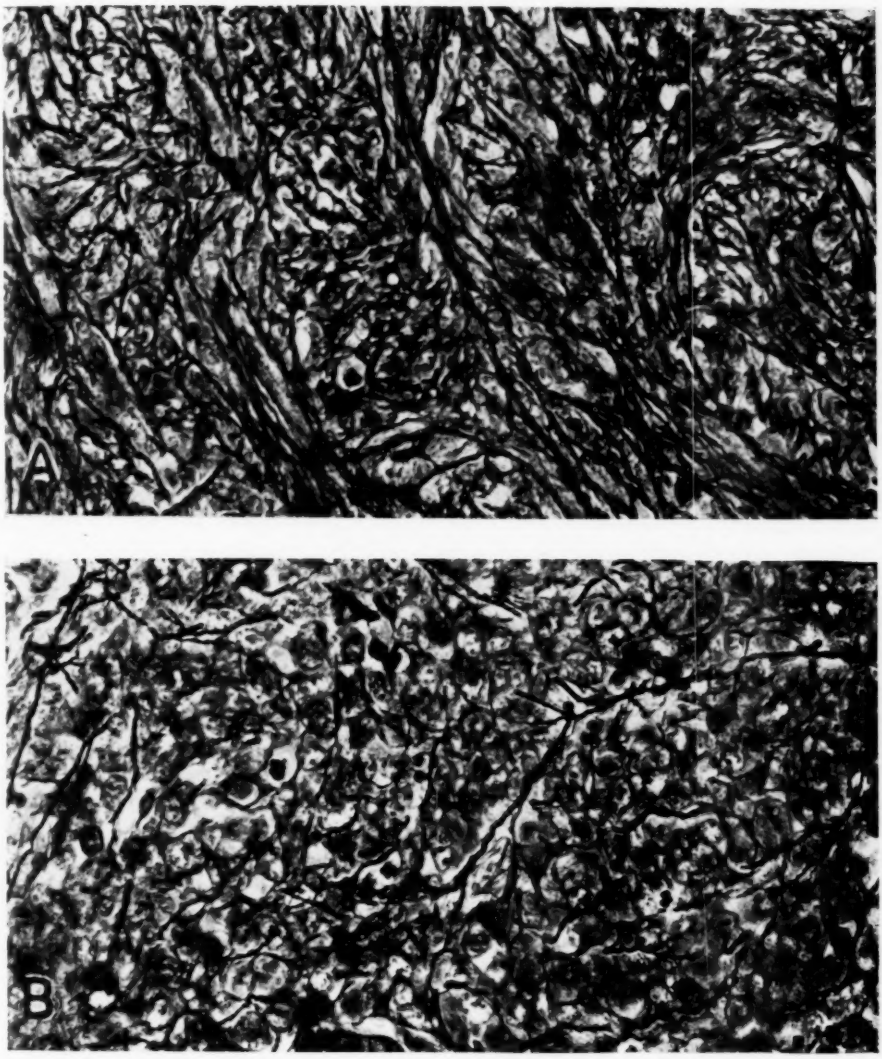

FIGURE 5.—*A*, Area considered to be fibrosarcoma in which the reticulum fibers may be seen to be intimately associated with and completely surrounding tumor cells. *B*, Arrangement of the reticulum in the more typical parts of the tumor of the later generations. Reticulum tends to surround groups of cells, but occasionally thin strands extend in between individual cells. Both *A* and *B* from tissue taken from the fourteenth-generation transplants, fixed in Zenker-formol, and stained with Laidlaw's stain for reticulum. × 490.

and insignificant. In these areas, mitotic figures were infrequent. With Mallory's phosphotungstic acid-haematoxylin stain, fibroglia fibers were seen. In sections stained for reticulum, the areas under consideration showed an abundance of reticulum with nearly every cell surrounded by fibers (fig. 5, *A*). Such areas are considered characteristic of fibrosarcoma. They were closely associated with the second type of tumor tissue, characterized by spherical to ovoid cells possessing slightly basophilic cytoplasm and large spherical, ovoid, or indented nuclei, which were relatively vesicular, and each of which showed at least one large, prominent basophilic nucleolus. Mitotic figures in these areas were extremely numerous. In such areas stained with haematoxylin and eosin, the connective tissue was not readily apparent and the cell boundaries were difficult to identify. With stains for reticulum, a striking difference was seen between these areas and those interpreted as fibrosarcoma. The reticulum was less abundant and tended to surround groups or cords of cells rather than individual cells (fig. 5, *B*). As was the case in the primary tumor, the reticulum surrounding groups of cells extended between some of the cells of the cords. With Mallory's phosphotungstic acid-haematoxylin stain, fibroglia fibers were not seen in most of the cells of these areas.

There were occasional giant cells which showed a relatively clear eosinophilic cytoplasm and several nuclei similar to those of the more common type of tumor cell. Subcutaneous transplants, pulmonary and lymph node metastases showed some straplike, elongated giant cells with fibrillated eosinophilic cytoplasm and a number of nuclei occasionally clumped together. No cross striations were visible. The nuclei of these cells were spherical and vesicular, and each possessed a large, prominent, basophilic nucleolus.

While osteoid tissue and true bone were found frequently in the early generations of transplants, and fibrosarcoma in the later generations, cartilage has never been identified.

CYTOPATHOLOGY[1]

Tissue from the primary tumor was not fixed for cytologic study, but it has been taken from all generations of transplants. In the first five generations, the Golgi material of the sarcoma cells regularly consisted of a closely woven network of rather fine osmiophilic threads arranged in spherical or ovoid form, similar to that described by Hill (*7*) in osteoblasts in tissue culture. It was juxtanuclear in position, but only rarely was it flattened against the nuclear membrane. It was located regularly between the nucleus and the developing osteoid tissue (fig. 6, *A*). The Golgi material of the cells in areas of fibrosarcoma appeared like that characteristic of fibroblasts and tumor cells of fibrosarcoma (*8*), being a rather open network of thin osmiophilic strands located near one pole of the nucleus. The Golgi material of the more common type of tumor cell in the later generations of transplants was similar to that of unspecialized cell types, being rather small and frequently flattened against the nuclear membrane (fig. 6, *B*).

The Golgi material of the giant cells in the earlier generations consisted of a thin band of osmiophilic material frequently completely surrounding the lobulated nucleus or nuclei (fig. 6, A). Under lower power of the microscope, it appeared to be a heavy precipitate outlining the nuclear membrane. The straplike giant cells present in later generations of transplants of the tumor possessed Golgi material which, when present, was quite different in character, being separated into small granular clumps of osmiophilic material associated with each of the several nuclei.

The mitochondria of the sarcoma cells of the earlier generations were filamentous, rather small, lightly staining, and moderate in number. They were much fewer than in the osteoblasts of normal mammalian embryos as figured by Deineka (*9*). The negative image of the Golgi material was frequently outlined by the mitochondria and was readily apparent in most of the tumor cells because of the more chromophilic character

[1] For details of the cytologic methods employed, see Dalton and Edwards (*6*).

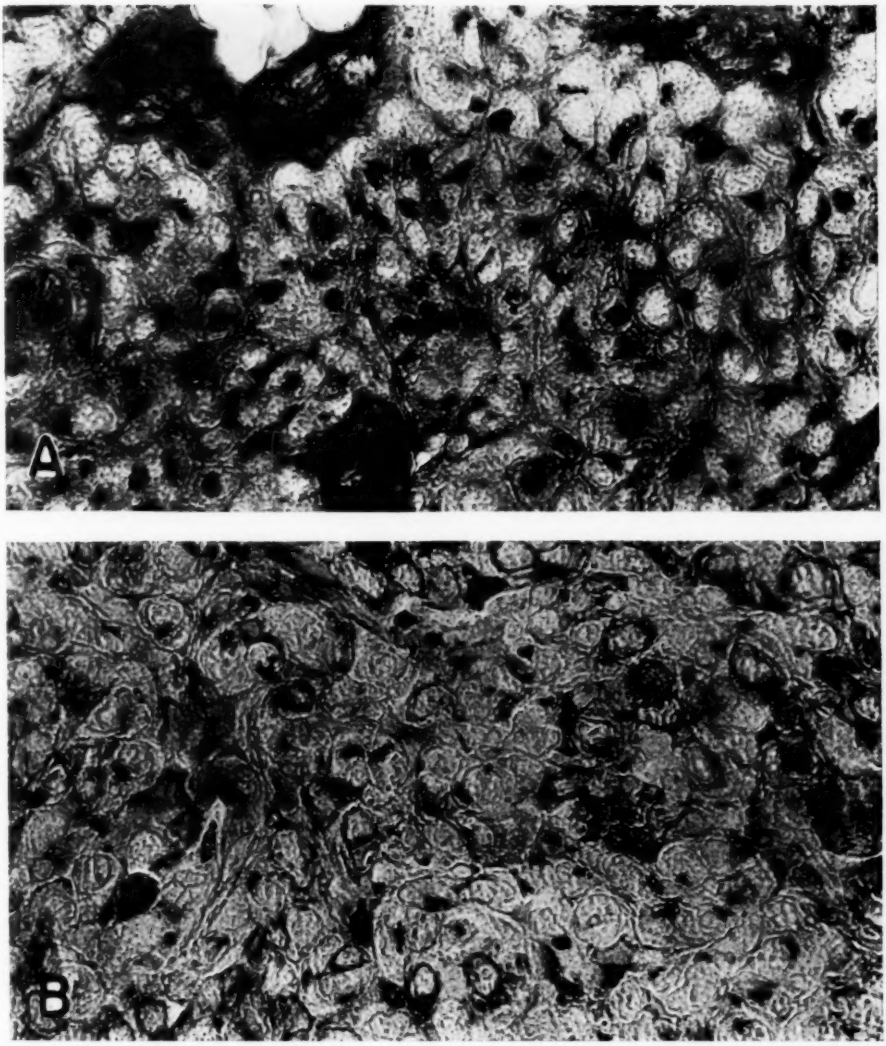

FIGURE 6.—*A*, Golgi material of the tumor cells and the tumor giant cells, second-generation transplant. *B*, Golgi material of the common type of tumor cell, fifteenth-generation transplant. Champy fixation and postosmication. × 1300.

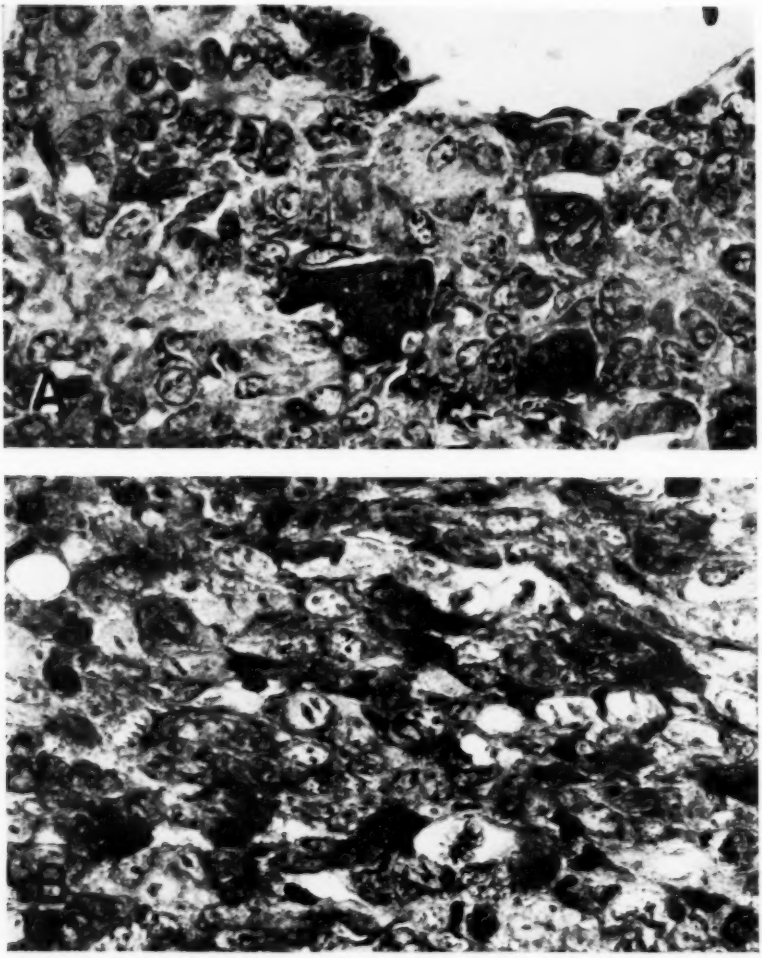

FIGURE 7.—*A*, Mitochondria of the tumor osteoblasts and the tumor giant cells in a third-generation transplant. The mitochondria of the tumor giant cells are very numerous, chromophilic spheres and beaded filaments. *B*, Mitochondria of the tumor cells in a fifteenth-generation transplant. Both *A* and *B* from material fixed in Regaud's fluid, and postchromated and stained with Heidenhain's iron haematoxylin. × 1300.

of the surrounding cytoplasm (fig. 7, *A*). The mitochondria of the giant cells were so numerous and so chromophilic that even the smaller giant cells (many of them the size of tumor osteoblasts) stood out sharply from the tumor cells.

The mitochondria of the tumor cells of the later generations were found in most cases to be similar in number and form to those in cells of earlier generations although occasionally cells with spherical mitochondria were seen (fig. 7, *B*). The mitochondria of the straplike giant cells were characteristically numerous, chromophilic, plump, short rods.

ACID AND ALKALINE PHOSPHATASE ACTIVITY

The activity of the enzymes in adult bone and in the early and later trans-

TABLE 1.—*Acid and alkaline phosphatase activity*

[5 cc. M/200 disodium phenylphosphate incubated at pH 4.6 and 9.5 for 1 hour at 37.5 C. with 0.5 cc. extract solution]

Tissue	Concentration of N in extract per cubic centimeter	Hydrolysis at pH 4.6	Q (acid)	Hydrolysis at pH 9.5	Q (alkaline)
	Milligrams	*Percent*		*Percent*	
Adult mouse bone	0.1	5	50	43	430
	.02	1	50	9	450
	.01	0		4	400
	.005	0		2	400
Average			50		420
Osteogenic sarcoma in mice, 3d generation	2.6	100		100	
	.52	68	131	100	
	.26	34	131	100	
	.13	17	131	100	
	.06	9	150	70	1,166
	.015	2	133	17	1,133
	.003	0		3	1,000
Average			135		1,100
Osteogenic sarcoma in mice, 17th generation	2.4	51	21	2	1
	.5	11	22	0	
	.25	5	20	0	
	.13	3	23	0	
	.06	0		0	
	.015	0		0	
	.003	0		0	
Average			21		<1

plants of the sarcoma was determined as before (*10*). The data are given in table 1.

The values of Q in the table refer to the ratio of percent hydrolysis to the concentration of total nitrogen in the extract. Within the range of concentrations studied, the splitting of the substrate is obviously a linear function of the dilution of the extract. No values of Q have been given when the percentage splitting is either 100 or 0 for the reason that 100-percent hydrolysis may have been achieved in less time than that of the incubation period chosen; the reason for not calculating Q at 0 percent splitting is obvious.

It is clear that there is a considerable difference in the activity of the phosphatases in the early and the later generation transplants of the sarcoma. The high values of the earlier generation transplants are characteristic of those observed in a wide variety of human osteogenic tumors (*11*, *12*). The low values of the later generation transplants on the other hand, practically coincide with those observed for transplanted mouse fibrosarcomas such as S37 and CR180 (*10*). In this connection, it is interesting to point out that whereas in the early generation transplants of the tumor the activity of alkaline phosphatase was much higher than that of acid phosphatase, a relation characteristic of osseous tissue, in the later generation transplants this relation was reversed, and the alkaline phosphatase activity nearly disappeared.

DISCUSSION

Apparently the difficulty of determining the exact site of origin of osteogenic sarcomas exists in animals as well as in man (*13*). While grossly an intimate fusion between the femur and the primary tumor was not apparent in the present case, histologic examination showed the presence of the tumor in the medullary cavity. For this reason the actual site of origin of the tumor is quite uncertain. It may have been entirely extra-osseous, invading the shaft and medullary cavity secondarily; it may have arisen from the margin of the medullary cavity (endosteum) and grown

outward through the Haversian or Volkmann canals to the surface of the bone; or it may have arisen from within the periosteum and grown in both directions, with penetration by permeation through the fibrous portion of the periosteum rather than by gross perforation.

When the material from the primary tumor was first examined, there was some question whether the neoplasm represented a true osteogenic sarcoma or an angio-endothelioma with coincident bone formation. The general picture of the cordlike arrangement of cells separated by wide blood spaces is often seen in human hemangio-endothelioma. This possibility was ruled out, however, when it was noted that the cells lining the blood spaces were not the tumor cells forming the cords but normal-appearing endothelium. The diagnosis of osteogenic sarcoma was definitely established when bone-forming pulmonary metastases were found in the first-generation transplants. That the primary tumor was sarcoma and not a benign lesion was made clear by the presence of tumor cells within the medullary cavity of the femur. According to the classification of Pybus and Miller (*4, 5*), the tumor could be considered a fibro-osteosarcoma with giant cells; however, we prefer to use the term "osteogenic sarcoma" which is more in keeping with the classification of bone sarcoma of the Codman registry of bone sarcoma of the American College of Surgeons.

Leriche and Policard (*14*) suggest that the presence of bone in all tumors in which it is found may be explained on the basis of metaplasia within the neoplasm. Intrinsic in their hypothesis is the idea that the normal or the neoplastic fibroblast, under proper environmental conditions (a local excess of calcium salts and/or phosphatase) may be stimulated to form osteoid tissue. Mallory (*15*) and Haining and Poole (*16*) present additional clinical evidence supporting this view, and there would be no question but that the presence of osteoid tissue and possibly true bone in various types of tumors are to be explained on this basis. However, Brunschwig and Harmon (*3, 17, 18*) present clinical cases and experimental evidence for which they feel the humoral hypothesis of Leriche and Policard offers an inadequate explanation. Subcutaneous transplants of fibrosarcoma were not found to form bone in the presence of excess calcium injected into the tumor mass. Further, fibrosarcoma introduced into the medullary cavity of long bones continues to form only fibrosarcoma while the normal periosteum, lifted by the growing tumor, is stimulated to form new bone. These authors are of the opinion that true osteogenic sarcomas continue to form bone both in the primary tumor and in any metastases derived from it. For this reason they prefer to use the term "osteoblastoma" to differentiate true osteogenic sarcomas from other bone-forming tumors

There is a third possibility which must be considered if all the clinical and experimental data are to be explained satisfactorily. It is suggested that the normal osteoblast is a definite cell type with its own special morphology and characterized by its function of forming osteoid tissue and that the malignant osteoblast retains this function to a variable degree. The extent to which this function is maintained in an osteogenic sarcoma appears to be inversely proportional to the rate of growth of the tumor. Thus, in a lesion with a moderate growth rate, osteoid-tissue formation would be expected in the primary tumor and in any metastases which might be produced. If a great increase in growth rate should occur, a decrease in the amount of osteoid tissue formed would be expected. Considerable evidence indicates the reasonableness of such a point of view. For example, a difference must exist between normal fibroblasts and osteoblasts since Hill (*7*) has shown that osteoblasts in tissue culture form osteoid tissue. Fibroblasts do not. Under the special conditions of a local excess of alkaline phosphatase and calcium salts, calcification does occur apparently in any area of connective tissue. Even under such conditions, there is no certainty that mature fibroblasts are responsible for the laying down of osteomucine, an essential part of true bone formation. On the other

hand, there is evidence that malignant osteoblasts are capable of forming osteoid tissue in areas where there is no reason to suspect an excess of alkaline phosphatase or calcium salts.

Murray (*1*) and Bashford (*2*) reported primary osteogenic sarcomas in mice, which continued to form bone and osteoid tissue in several generations of subcutaneous transplants. In the present instance osteoid tisue was found in several generations of subcutaneous transplants, and in addition numerous pulmonary metastases and one metastasis to the liver were found in which osteoid tissue was present. However, the statement of Foulds (*19*) that bone formation continues in transplants should not be taken to mean that it necessarily continues indefinitely.

There is apparently a general tendency for tumor cells which possess a special function to lose it after a variable number of transplantations if during that time there is an increase in growth rate. In the present case osteoid tissue was not formed after the sixth generation, and this change was coincident with a rapid increase in growth rate and the loss of an originally high alkaline phosphatase content. The history of a recently reported, transplantable, adrenal cortical tumor (*20*) supplies confirmatory evidence. In the early generations of this tumor, cells containing lipid droplets and resembling the cells of the zona fasciculata of the normal adrenal gland were numerous. In later generations in which the growth rate had greatly increased, such cells were no longer in evidence. Apparently expression of special functions is not to be expected in cells which have achieved a rapid growth rate. However, it would not necessarily follow that, should such cells resume a slow rate of growth, they would again exhibit their special function.

It is suggested, therefore, that while many bone-containing tumors occur in which the tumor cells may be stimulated to participate in osteoid-tissue formation by extrinsic (extracellular) factors, true osteogenic sarcoma is characterized by the presence of a tumor-cell type possessing the intrinsic capacity to produce bone or osteoid tissue. As stated by Foulds, these cells have the capacity to form bone but do not always exercise it.

Kolodny (*13*) has suggested that osteogenic sarcoma cells may differentiate from small spindle-shaped cells, forming little intercellular substance, into more or less typical osteoblasts forming relatively large amounts of "near osteoid" material and that—

> Ossification of the intercellular substance in osteogenic sarcoma is the apex of differentiation which may be reached by the tumor cells and intercellular substance.

In view of the history of osteogenic sarcomas in transplants, it is suggested that while Kolodny's interpretation may be correct, the possibility must also be entertained that a cell may acquire malignant properties at any stage in its evolution into the adult osteoblast and that, coincident with a gradually increasing growth rate, the capacity to form intercellular bone matrix is either lost or inhibited. In this view, the presence or absence of bone or osteoid tissue in an osteogenic sarcoma would depend upon the stage of differentiation of the cell at which it became malignant and upon the extrinsic factors that determine or control the growth rate of the tumor cells. In the present case and in accordance with the view just presented, the changes in cytology and histology and the loss of phosphatase in the later generations of transplants are more or less what might be expected in a tumor that has acquired a rapid growth rate.

SUMMARY

The history, pathology, cytology, and phosphatase activity of a spontaneous, transplantable, osteogenic sarcoma are reported.

The primary tumor, the early generations of subcutaneous transplants, and the pulmonary metastases arising from them consisted of malignant osteoblasts, osteoid tissue, and true bone. The early generations of transplants were char-

acterized by a moderate growth rate and the possession of a high alkaline phosphatase activity.

With a rapid increase in growth rate in later generations, the high alkaline phosphatase activity and the capacity of forming osteoid tissue characteristic of the early generations were either inhibited or lost. Coincident with the increase in growth rate, early metastases to regional lymph nodes as well as to the lungs were noted.

The significance of these observations as they relate to the question of the cell of origin in osteogenic sarcoma is discussed.

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IMPORTANCE OF GENETIC INFLUENCE ON THE OCCURRENCE OF MAMMARY TUMORS IN VIRGIN FEMALE MICE

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This mammary-tumor study was made with the two inbred mouse strains, A and C3H, both of which show a high incidence of mammary tumors in the breeding females. Although most of the genetic and foster-nursing work on mammary tumors has been done by using high- and low-mammary-tumor strains, with their various types of hybrids, there appeared to be certain distinct advantages that could be gained by using these two high-mammary-tumor strains.

The primary purpose of the project was the analysis of factors causing a difference in tumor incidence of the virgin females of the two strains. Both strains do possess the milk agent and both display a high degree of inherited susceptibility. In strain C3H, however, mammary tumors develop in a high percentage of both breeding and virgin females; but in strain A, while such tumors develop in a high percentage of the breeding females, the incidence among the virgin females is low. Thus, the question arose: Do these strains differ fundamentally in the genic complex influencing mammary-tumor development or does the milk agent differ in the two strains? The following data show that the strains differ in genic complex, but there is no conclusive evidence of difference in the inciting agent of the milk.

MATERIALS AND METHODS

Strain A and strain C3H mice of sublines maintained at the National Cancer Institute were used. Both of these strains have been used extensively for mammary-tumor work (*1-12*).

Two sublines of strain A were used, one in the reciprocal crosses and the other in the foster-nursing work. There was no evidence, however, that these two sublines differed. Both were descendants of mice obtained from Dr. J. J. Bittner's strain A colony at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and have been separated from his colony for 5 years. In general, the mammary-tumor incidence for breeding females of the strain is high; percentages ranging as high as 90 and above have been published (*5-8*). The incidence of mammary tumors in the virgins is low. Bittner (*6*) has published an incidence of 4.9 percent, with an average tumor age of 18.5 months.

The strain C3H mice were descendants of animals obtained in 1930 from Dr. L. C. Strong. Brother-sister generations total over 30 since the animals were received in this laboratory. The strain is highly susceptible to the development of mammary tumors, as is demonstrated by the following data given by Andervont (*1*) for this particular subline:

> Of 1,588 breeding females, 91.37 percent developed spontaneous mammary-gland tumors at an average age of 8.58 months. Of 350 virgin females, 97.43 percent developed spontaneous mammary-gland tumors at an average age of 10.43 months.

In these experiments six different groups of virgin females were produced: two from reciprocal crosses between the parent strains, two from reciprocal foster nursing between the parent strains, and two were litter-mate controls for the foster-nursed groups.

In the reciprocal crosses 9 strain A females were mated to strain C3H males, and 13 strain C3H females were mated to strain A males. These two crosses produced F_1 hybrids, half of whose chromatin was derived from strain A and half from strain C3H; but the group whose mothers were strain A females received strain A milk factor and possible intra-uterine influence, while the group whose mothers were strain C3H females received these extrachromosomal influences from strain C3H.

Although mammary tumors developed in but 4 of these 9 strain A mothers at an average tumor age of 10.5 months, this is not considered a fair test of the susceptibility of these females, for the

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other 5 were discarded at an early age because of an infestation of the mite, *Myobia musculi*. Of a total of 348 breeding females of this particular subline, mammary tumors have appeared in 73 percent at an average age of 9.79 months, the average age at death of nontumorous females being 9.97 months. Mammary tumors developed in all 13 of the C3H mothers at an average age of 7.8 months.

In the reciprocal foster-nursing project, 31 strain A and 31 strain C3H females were mated to their male litter mates. After they became pregnant, they were isolated and observed twice daily for the birth of the litters. When a C3H litter and an A litter were found, half of each was exchanged, thus producing strain C3H young foster nursed by strain A and strain A young foster nursed by strain C3H, with littermate controls for each group. In no case did the foster-nursed young stay with their mothers longer than 17 hours.

Both groups of mothers used in the foster-nursing experiment displayed a high degree of susceptibility. Mammary tumors developed in all of the 31 C3H females at an average age of 8.9 months, and in 28, or 90.3 percent, of the strain A females at an average age of 9.6 months.

In view of some recent work of Bittner (*5*) which suggests that there may be an increase in tumor incidence among animals of later litters over that of earlier litters, it should be noted that the animals of the reciprocal crosses were limited to those of the first and second litters. In the reciprocal foster-nursing work, litters ranging from the first to the fifth were used. The possibility, however, that litter number produced a variation in the results here was greatly curtailed by the use of littermate controls.

Only the female mice produced by means of the procedures described were included in the experiments. All those of the six groups (four experimental and two control) were kept as virgins. They were reared in comparable environments, being housed in wooden cages, eight animals to each cage, and given an unlimited supply of tap water and Purina dog chow. At biweekly intervals they were observed for the presence of tumors; and after the tumors appeared, the animals were necropsied and the diagnoses confirmed by histologic sections.

RESULTS AND DISCUSSION

As noted previously, the primary purpose of these experiments was to ascertain whether or not the low tumor incidence among the virgin females of strain A as compared with the high tumor incidence among the virgin females of strain C3H was due fundamentally to a difference in genetic constitution of the two strains or to a difference in the milk agent. The data in table 1 show that the variation was owing in part to genetic factors. There was a slight suggestion that the milk agent of the two strains might also differ, but this evidence was far from conclusive.

In none of the 22 strain A females that nursed their own mothers did mammary tumors develop, although these females lived to an average age of 16.36 months. Of the 32 litter mates of these strain A females which were foster nursed by strain C3H females, mammary tumors arose in 5, or 16 percent, and these tumors appeared early, at an average age of 10.6 months. This fact, in itself, suggested a possible difference in the milk agent of strain A as compared with that of strain C3H. The significance of this difference, however, is minimized when one considers that experience with strain A mice in general has shown that tumors can be expected to arise in approximately 5 percent of the virgin females.

Although little, if any, difference was produced by introducing strain C3H mammary-tumor agent into strain A, a decided increase in tumor incidence was produced by introducing chromatin from strain C3H. Tumors arose in 91 percent of the 45 F_1 hybrid females whose mothers were strain A and contributed strain A extrachromosomal influences but whose fathers were strain C3H. Thus, the introduction of the strain C3H chromatin from these fathers raised the tumor incidence from the very low per-

TABLE 1.—*Occurrence of mammary tumors in the different types of virgin females*

[Numerator—mice developing tumors
Denominator—mice dying without tumors]

Strain of mother	Strain of father	Nursed by strain—	Number of mice	Time in months																		Total	Incidence of mammary tumors	Average tumor age	Average age of mice dying without tumors
				7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24				
																							Pct.	*Mos.*	*Mos.*
A	A	A	22	---	---	0/1	---	---	0/2	---	---	0/2	0/6	0/3	0/2	0/6	---	---	---	---	---	0/22	0	-----	16.36
A	A	C3H	32	---	---	1/0	1/0	2/1	1/0	0/3	---	---	0/5	0/4	0/9	0/5	---	---	---	---	---	5/27	16	10.60	16.85
A	C3H	A	45	1/0	1/0	2/0	2/0	2/0	---	2/0	3/0	5/0	3/0	4/0	6/0	4/0	2/1	1/1	1/2	1/0	1/0	41/4	91	15.80	21.25
C3H	C3H	C3H	29	1/0	2/0	7/0	3/1	5/0	3/0	2/0	1/0	---	3/0	---	0/1	---	---	---	---	---	---	27/2	93	10.93	14.00
C3H	C3H	A	35	1/0	2/0	4/0	4/0	8/0	5/0	3/0	2/0	1/0	2/0	0/1	1/1	---	---	---	---	---	---	33/2	94	11.48	17.50
C3H	A	C3H	36	---	0/1	0/1	6/0	2/1	4/0	4/0	5/0	1/0	---	4/0	1/1	---	3/2	---	---	---	---	30/6	83	13.77	14.33

centage of the strain A females to a high percentage not significantly different from that of the strain C3H females.

The tumor incidence was not significantly altered in the strain C3H females by permitting them to be foster nursed by strain A females. The incidence in this foster-nursed group of 35 strain C3H females was 94 percent as compared with 93 percent in their 29 litter-mate controls that were nursed by their C3H mothers. Furthermore, the introduction of strain A chromatin by mating the strain C3H females to strain A males did not lower the tumor incidence significantly. In the resultant 36 F_1 hybrids, tumors arose in 30, or 83 percent.

When the average tumor age was considered, however, significant differences were found between some of these groups not showing real differences in tumor incidence. Of the four groups with high tumor incidence, the lowest averge tumor age was 10.93 months, that of the control strain C3H females. The introduction of the milk agent from strain A resulted in an average tumor age of 11.48 months, which was not a significant increase over the control C3H group. But by introducing chromatin from the strain A fathers, the average tumor age was increased to 13.77 months in the F_1 hybrids produced. In comparison with the control strain C3H females, the difference was significant (t=3.83; P<0.01).[1] Furthermore, in comparison with the C3H females foster nursed by strain A females, the difference was also significant (t=3.176; P<0.01).[2] The average tumor age was again increased by introducing both strain A chromatin and strain A extrachromosomal factors, as was done in the case of the F_1 hybrids resulting from the mating of strain A females with strain C3H males. In this group, the average tumor age was 15.8 months as compared with that of 13.77 months for their reciprocal hybrids. Here the difference was at least borderline in significance (t=2.344; P between 0.01 and 0.02).[3]

One of the incentives for the project was the hope of producing an F_1 hybrid more susceptible than even the strain C3H. Previous work (*2*) showed that when two low-mammary-tumor strains, C57 Black and I, were crossed, the resultant F_1 hybrids were more susceptible than either parent strain. However, in the present experiments the F_1 hybrids were intermediate in susceptibility between the parent strains although they were more like strain C3H than strain A. Bittner (*7*) reciprocally crossed two high-tumor strains, A and D,[4] and found that the F_1 hybrids resembled the maternal strain but in neither case exceeded either parent strain when both percentage of tumors and tumor age were considered, although in both groups of

[1] These probabilities were calculated with the use of the t statistic of Fisher (*13*).

[2] See footnote 1.

[3] See footnote 1.

[4] Referred to as strain dba in Mouse Genetic News, November 1941, George D. Snell, editor.

hybrids the incidence was slightly higher than in either parent strain.

It is now well recognized that at least three influences are involved in mammary-tumor development: (1) The genetic constitution; (2) the hormonal stimulation; and (3) the inciting agent usually transferred by way of the mother's milk.

Murray (*10*) has recently emphasized the importance of the difference in concentration of the extrachromosomal stimulus and has pointed out that—

> the amount of mammary carcinoma occurring in a stock or cross, may be explained on the basis of: (1) The concentration of the extrachromosomal stimulus received. (2) The resistance of the physiological system of the host to this stimulus.

The data in the present paper offer a meager suggestion that these strains do differ in the stimulus occurring in the milk, but the evidence is inconclusive.

The interrelation of the genetic constitution and the hormonal stimulus is evident, but the mechanism is not clear. Mammary tumors arise in but few strain A virgin females as compared with the high incidence among strain C3H virgin females, yet as a result of breeding, the tumor incidence of the strain A females is high. Furthermore, the injection of estrogen into strain A virgin females results in a high percentage of tumors (*9*, *11*, *12*). It might be suspected that the strain C3H virgin females had more tumors because of greater hormonal stimulus, but this has not been demonstrated. These experiments show that the fundamental differences of the two strains lie in the genetic factors. It may be that these genetic factors are affecting the tumor incidence by influencing hormone production, or they may be influencing susceptibility to the hormone, or they may be acting in both ways.

SUMMARY

Two inbred strains of mice, A and C3H, were studied. Both are high-mammary-tumor strains but differ in that whereas both the breeding and virgin females of strain C3H have a high incidence of mammary tumors, only the breeding females of strain A have a high incidence. Few mammary tumors develop in the virgin strain A females.

Reciprocal crosses between the two strains demonstrated that this difference was due largely to a difference in genic complex influencing tumor development. In degree of susceptibility to mammary tumors, the F_1 hybrid virgin females were intermediate between the parent strains although more like the parent strain C3H.

Reciprocal foster nursing between the two strains did not give conclusive evidence of a difference in the mammary-tumor agent in the milk of the two strains.

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EFFECT OF AMINO ACIDS ON THE INDUCTION OF LEUKEMIA IN MICE

By JULIUS WHITE, *senior biochemist*, G. BURROUGHS MIDER,[1] and WALTER E. HESTON, *geneticist*, *National Cancer Institute, National Institute of Health, United State Public Health Service*

It has been reported (*1*, *2*) that strain dba, subline 212, mice fed a low cystine diet from weaning and painted 20 times with an ether solution of methylcholanthrene developed leukemia to the extent of 10 percent. However, 90 percent of the mice on the same diet supplemented with cystine developed leukemia.

More recently (*3*) it has been shown that strain C3H virgin female mice ingesting the low cystine diet from weaning developed no mammary-gland tumors during 22 months. Of the litter mates which consumed the cystine supplemented diet, 97.6 percent had mammary-gland tumors at an average age of 8.4 months.

The question arose whether the reduction in the incidence of leukemia in strain dba mice was associated specifically with the cystine content of the diet or whether this change was characteristic of other dietary inadequacy or of the restriction of any essential amino acid. To study these possibilities three experiments were undertaken.

EXPERIMENTAL PROCEDURE

EFFECT OF LACK OF LYSINE

To determine whether restriction in another essential amino acid would result in the same reduction in the occurrence of leukemia as brought about by the low cystine diet, a diet low in lysine was devised:

	Percent
Gliadin[1]	18.0
Cornstarch	50.0
Salts (modified Osborne-Mendel)	4.0
Crisco	20.0
Cod-liver oil	3.0
Ryzamin B No. 2 (Burroughs-Wellcome)	5.0
Total	100.0

[1] Huron Milling Co., Harbor Beach, Mich.

On this diet weanling strain dba mice were able to maintain their weight but were unable to grow. The substitution of 0.62 percent *d*-lysine monohydrochloride (0.5 percent free *d*-lysine) for 0.62 percent of the starch in the diet resulted in growth which was equivalent to that of mice fed the stock Purina dog-chow ration.

Strain dba mice used in this experiment were divided at weaning into two groups: one was placed on the low lysine diet and the other on the same diet supplemented with *d*-lysine. The mice were painted 20 times with a 0.2-percent methylcholanthrene solution in diethyl ether in the manner previously described (*2*) and observed until death. The average weight of the mice was 15.9 gm. when started on the experiment. Sixty days later those on the low lysine diet weighed 15.5 gm., while those on the lysine-supplemented diet averaged 27.2 gm.

The data are presented in table 1. The incidence of leukemia was the same whether or not the mice were given added lysine. Inasmuch as the lack of the essential amino acid lysine did not influence the latent period or the incidence of leukemia, it seemed probable that cystine played some role in tumor formation, perhaps not identical with its function as an amino acid essential for normal growth. Whether the limitation of other essential amino acids would produce effects similar to that of either cystine or lysine will be determined in subsequent investigations.

EFFECT OF THE ADDITION OF GELATIN TO A LOW CYSTINE DIET

Included in table 1 are the results of an experiment in which a group of strain dba mice was placed on the low cystine diet in which 5 percent of the starch was replaced by gelatin. Gelatin contains 0.2 percent cystine and no methionine (*6*), and the addition of this substance, therefore, increased the protein content without markedly increasing the sulfur-containing amino acids. The purpose of this procedure was to determine whether added protein nitrogen would influence the incidence of leukemia induced with

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TABLE 1.—*Effect of diet on the response of strain dba mice to painting with methylcholanthrene*

Diet	Mice	Paintings	Mice with leukemia[1]			Mice with sclerosis[2]		
			Number	Percent	Mean latent period	Number	Percent	Mean latent period
	Number	*Number*			*Days*			*Days*
Dog chow[3]	[4]40	20	36	90	99	0		
High cystine[3]	40	20	36	90	101	0		
Low cystine[3]	40	20	4	10	127	32	80	144
Low cystine+gelatin	38	20	12	32		2	5.3	
High lysine	36	20	28	78	101	2	5.6	
Low lysine	36	20	26	72	104	2	5.3	
High cystine[5]	39	40–70	35	90	81	0		
Low cystine+methionine	20	40–70	19	95	73	0		

[1] Pathologic diagnosis described by Mider and Morton (*3*).
[2] Pathologic diagnosis described by White and Mider (*1*).
[3] Data taken from White, Mider, and Heston (*2*).
[4] Number of mice in each group 60 days after the start of the experiment.
[5] Data taken from White and Mider (*1*).

methylcholanthrene. It would have been expected that, if the concentration of cystine or methionine was the controlling factor in the production of leukemia under the experimental procedure employed, the addition of 5 percent of gelatin should have had no effect on the incidence of leukemia except that which would be contributed by the amount of cystine in the gelatin. The original low cystine diet contained only enough sulfur-containing amino acids for the maintenance of the weight of weanling mice. Therefore, even the small amount (10 mg. per 100 gm. of diet) of cystine contributed by the gelatin would influence the incidence of leukemia if cystine played such a role. The average weights of the mice at the start of this experiment and 60 days later were 16.4 and 15.7 gm., respectively. The data (table 1) indicate that the addition of the gelatin resulted in an increase in leukemia to 32 percent. Mice maintained on the low cystine diet showed an incidence of 10 percent leukemia. This increase in leukemia from 10 to 32 percent seems to be in the range that small increases in dietary cystine might be expected to give. The data suggested that added nitrogen from gelatin did not influence the incidence of leukemia except for the effect of the cystine it contained.

Previous experiments showed that 80 percent of the mice fed the low cystine diet developed sclerosis of the larger blood vessels. However, this experiment showed that the addition of 5 percent of gelatin decreased the incidence of this lesion to 5.3 percent.

COMPARISON OF THE EFFECT OF METHIONINE AND CYSTINE

Cystine in the diet of the young rat can be completely replaced by methionine for purposes of growth. To determine whether or not methionine could function in a manner similar to cystine in methylcholanthrene-induced leukemia in mice, a group of strain dba mice were fed the low cystine diet (*4*) supplemented by 0.5 percent *dl*-methionine. They were painted in the manner previously described (*1*) with a 0.2-percent solution of methylcholanthrene in diethyl ether on alternate days until death. This resulted in from 40–70 applications. The average weights of the mice were 18.0 gm. at the start of this experiment, and 21.8 gm. 60 days later. The data are also presented in table 1. For comparison, data on the production of induced leukemia in mice on the low cystine diet supplemented with 0.5 percent *l*-cystine are reprinted (*2*). The results of this experiment indicated that methionine was fully as effective as cystine in the production of leukemia induced by painting strain dba mice with methylcholanthrene.

SUMMARY AND CONCLUSIONS

Strain dba mice painted with methylcholanthrene developed essentially the

same incidence of leukemia on diets with or without adequate lysine.

The data suggest that cystine played a role in the development of induced leukemia perhaps not associated with its properties as an essential amino acid for growth, but with some other undetermined property.

The addition of gelatin to a low cystine diet altered the occurrence of leukemia following methylcholanthrene painting from 10 to 32 percent. This increase was attributed to the small amount of cystine present in gelatin.

The incidence of leukemia in methylcholanthrene-painted mice was increased to the same extent when the low cystine diet was supplemented with methionine as with cystine.

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EFFECT OF DIETHYLSTILBESTROL ON MAMMARY TUMOR FORMATION IN STRAIN C3H MICE FED A LOW CYSTINE DIET

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It has been shown that the Andervont subline of strain C3H virgin female mice, which under normal circumstances show a mammary-tumor incidence of 97.43 percent (*1*), when maintained from the time of weaning on a diet low in cystine, developed no mammary tumors (*2*). Gross mounts of mammary tissue of these mice maintained on the low cystine diet showed that very little growth of this structure had taken place, regardless of age. The mounts showed only a rudimentary duct system (*2*), indicating that the effect of the ovarian estrogenic hormone in bringing about growth of the mammary-duct system had been absent.

The full growth of mammary-gland tissue has been shown to be under the control of several hormones (*3, 4*). However, the following considerations made it seem most probable that lack of the estrogenic hormone was primarily responsible for the lack of mammary-tumor formation in mice fed a low-cystine diet:

(1) It is known that estrus exerts a powerful influence on mammary-gland development. Turner and Gomez (*5*) described the elongation of the end-buds during estrus, and their partial regression near the next proestrus stage. By this means the extent and complexity of the duct system was increased until the mouse was about 70 days of age (*6*), when it reached its full virginal development.

(2) It seemed probable that estrus was absent in the mice fed the low cystine diet. Pearson (*7*), working with rats, stated that if the diet contained 10 percent casein, estrus was not disturbed, whereas if the casein was reduced to 5 percent, there was, in most cases, a complete cessation of estrus, although in a few instances prolonged or irregular cycles were shown. Limited observations of strain C3H mice fed a low cystine diet, by means of vaginal smears and by the observed lack of corpora lutea in the ovaries at autopsy, confirmed the impression that a diet low in casein, and thus limited by the sulfur-containing amino acids, had the same effect upon estrus in mice.

The incidence of spontaneous mammary tumors was essentially the same (*1*) in virgin and breeding females in the Andervont subline of strain C3H mice. The development of the mammary gland to the extent that was primarily due to the estrogenic hormone of the ovary seemed to be the factor the lack of which prevented the formation of tumors in mice ingesting the low cystine diet.

This paper reports the incidence of spontaneous mammary-gland tumors in a subline of strain C3H female mice under dietary regimes identical with those previously used, but each mouse carried a small subcutaneously implanted pellet of diethylstilbestrol, an estrogenic compound (*8*).

Strain C3H virgin females, supplied by Dr. H. B. Andervont, of this Institute, were divided into three groups at weaning. One group was fed Purina dog chow, the second group a low cystine diet (*9*), and the third group the low-cystine diet supplemented with 0.5 percent *l*-cystine. Pellets of 25 percent diethylstilbestrol in cholesterol, which weighed an average of 1.6 mg., were implanted subcutaneously at the same time. The mice were kept two in a cage, and a weekly record of body weights was made. At each weighing the mice were examined for mammary-gland tumors, and the presence of the pellet was confirmed. If the pellet was not found by palpation, daily vaginal smears were taken, and as soon as continuous cornification ceased another pellet was implanted. Usually there was an interval of about a week between the time the pellet was not felt and the end of its activity. If the cornified smear continued much beyond this time, a more thorough search for the pellet usually revealed it, either in an

unexpected location or strongly encapsulated and attached to the underlying muscles, so that it could be identified only with difficulty.

When a tumor was observed, a note of it was made in the record, and the mouse allowed to live a week or two longer. All animals were necropsied. Sections were prepared of suspected tumors, and diagnosis of mammary-gland tumors was made by Dr. A. B. Eschenbrenner, of this Institute.

RESULTS AND DISCUSSION

The results are summarized in table 1. Whereas none of the mice fed the low cystine diet without diethylstilbestrol pellets showed mammary tumors, 44.7 percent of the mice with pellets developed tumors at an average age of 11 months. The pellets weighed an average of 1.6 mg. (range 1.3—2.0 mg.) when implanted and 1.2 mg. (0.8—1.7 mg.) when recovered at autopsy. No particular care was taken in removal to retain the softened exterior of the pellet, so that the difference in weight of the implanted and recovered pellet was probably in excess of the amount absorbed. However, if 0.4 mg. of the pellet was used, 25 percent of this was diethylstilbestrol (*10*), or an average of 0.1 mg. over 11 months. The fact that the rate and total incidence of tumor formation in mice fed either chow or the high cystine diet was the same whether or not they bore a pellet of diethylstilbestrol, indicated that the amount of estrogen administered did not influence tumor formation in normal mice. Moreover, the tumors in all the mice with diethylstilbestrol pellets were normally distributed among the mammary glands and not concentrated in those glands nearest the pellets. For this reason it was felt that the increase in mammary tumor in mice with pellets, on the low cystine diet, was ascribable to substitution of the estrogenic action of diethylstilbestrol for the hormone produced in normal mice by the developing follicles in the ovaries.

The fact that only about half of the mice implanted with pellets and fed the low cystine diet developed tumors might have been due to the operation of factors other than a lack of the estrogenic hormone in mice on the low cystine diet. Progesterone, the hormone of the corpus luteum, which would be produced during a part of every normal estrous cycle, was, of course, absent in these pellet-implanted mice, since no ovulation was caused by diethylstilbestrol. The combined action of progesterone and an estrogen was shown to be necessary (*11*) for the proliferation of the lobule-alveolar system in the mammary gland of the mouse. Furthermore, Gomez and Turner (*12*) demonstrated in rats the necessity of a hormone of the anterior portion of the pituitary gland for growth

TABLE 1.—*Effect of subcutaneously implanted pellets of diethylstilbestrol upon spontaneous mammary tumors in strain C3H virgin female mice*

[Numerators=number of mice developing tumors
Denominators=number of mice dying free of tumor]

Group of mice and diet	Effective number of mice	Mice developing tumors or dying free of tumors according to age (in months)																Tumor incidence	Average tumor age
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	Total		
Mice without pellet:																		*Pct.*	*Mos.*
Chow[1]	350	3/0	11/1	20/0	35/0	63/0	51/0	56/1	41/1	26/1	13/1	10/2	5/0	6/0	1/1	0/1	341/9	97.43	10.43
High cystine[1]	42	0/0	11/1	5/0	9/0	6/0	2/0	4/0	3/0	1/0	---	---	---	---	---	---	41/1	97.61	8.36
Low cystine[2]	45	0/0	0/0	0/0	0/0	0/0	0/0	0/3	0/2	0/0	0/1	0/0	0/2	0/1	0/0	0/0	0/9	.00	-----
Mice with pellet:																			
Chow	27	0/0	4/0	3/0	2/0	3/0	4/0	3/0	3/0	1/0	0/1	[3]	---	---	---	---	23/1	95.83	9.13
High cystine	28	0/1	3/1	4/0	7/0	3/0	1/0	5/0	0/0	0/0	0/0	[3]	---	---	---	---	23/2	92.06	8.43
Low cystine	40	0/0	0/4	2/3	2/2	3/1	1/1	1/1	1/1	2/1	3/1	1/1	0/1	1/4	[4]	---	17/21	44.74	11.18

[1] Data from Andervont (*1*).
[2] Data from White and Andervont (*2*).
[3] 3 alive at the end of 14 months.
[4] 2 alive at the end of 17 months.

of the mammary gland in response to progesterone and an estrogen. If mice require this mammogenic pituitary hormone, perhaps the amount of it produced was altered by the low cystine diet, in which case diethylstilbestrol would not be completely effective.

The average weight of the female mouse of this strain is about 22 to 23 gm. upon reaching maturity (at 7 to 8 weeks). The average weight at weaning (age approximately 1 month) is 16 to 18 gm. At this stage, the formation and deposition of new protein in muscles and organs is in its last stages. The animals fed the low cystine diet, whether or not they bore a stilbestrol pellet, maintained their weights throughout the experimental period. The fact that the mice given diethylstilbestrol nevertheless developed mammary-gland tumors indicated that restriction of further growth was not the primary controlling factor in the prevention of mammary tumors by the low cystine diet.

SUMMARY

In strain C3H virgin female mice fed a low cystine diet spontaneous mammary-gland tumor formation was completely prevented. This suppression was shown to be due, in part at least, to lack of estrus, since diethylstilbestrol pellets implanted subcutaneously raised the incidence of tumor formation from 0 to 44.7 percent.

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BLOOD PROTEOSE [1] AND CANCER [2]

By RICHARD J. WINZLER,[3] *research fellow,* and DEAN BURK, *senior chemist,* with the *technical* assistance of MARIE HESSELBACH, *National Cancer Institute, National Institute of Health, United States Public Health Service*

A test for cancer based on the presence of increased amounts of protein split-products in the serums or bloods of cancer patients has been proposed and partly developed for clinical use by Brdicka, Mayer, Waldschmidt-Leitz, and others (*2–6*). The method involves the precipitation of the serum proteins with sulfosalicylic acid, followed by polarographic analysis of the filtrate in the presence of hexammine cobaltic chloride and ammonium hydroxide. Under these conditions proteins and high-molecular-weight polypeptides containing cysteine or cystine give a highly specific current wave at the dropping mercury electrode because of the catalytic evolution of hydrogen by the sulfhydryl and disulfide groups.[4] Sulfosalicylic acid-deproteinated filtrates of serums of cancer patients ordinarily give higher polarographic waves than do normal serums, even after dialysis, indicating that they contain more high-molecular-weight protein split-products. A review of the literature (*4, 6, 7, 8, 9, 10, 11*) reveals that 93 percent of the serums of some 1,400 cancer patients have given polarographic filtrate waves distinctly higher than normal. As Brdicka and others have pointed out, however, the test as now carried out is not specific for cancer, since high polarographic filtrate waves also result from certain other diseases, particularly those involving pyogenic infections. Thus, of about 200 such patients, 69 percent gave positive tests. A brief account of the application of polarography to serological detection of cancer is given by Kolthoff and Lingane (*12*).

Although the polarographic test fails in its present state to be specific for cancer, there are, nevertheless, various potential chemical, biologic, or immunologic methods of analysis for proteose other than the polarographic test; and certain of these, especially in combination with the polarographic test, might yield specific differentiations between cancer proteoses and other proteoses.[5] The present paper is not immediately concerned with such possible differentiations but lays a certain basis for studies directed to this end. The material responsible for the polarographic filtrate waves in the blood of normal rats has been isolated as a sulfosalicylate conjugate and partially characterized as to chemical composition. The relationships of the polarographic filtrate wave analysis to earlier studies of polypeptidemia and to the polysaccharide C-precipitable serum protein occurring in certain pathologic bloods have also been investigated.

Little polarographic work has been reported on animal bloods or serums heretofore. Klatt, Rusch, Dirksen, and Meloche (*16*) studied the protein waves of serums of rats, mice, and humans, Crossley and co-workers (*17, 18*) studied the filtrate waves of serums of normal and pneumococcus-infected dogs. In the present work, the proteose filtrate waves of bloods as well as serums of a variety mammals, including man, have been

[1] The term "proteose" is used in this paper to designate non-heat-coagulable, sulfosalicylic acid-soluble, non-dialyzable, protein split-products precipitable by saturated ammonium sulfate. Although what constitutes a proteose is not better defined in the protein field, the chemical and physical properties listed herein would appear to justify the use of the term to cover certain blood components studied and to define further this class of compounds.

[2] A brief summary of the biologic aspects of this work was presented at the thirty-fifth annual meeting of the American Association for Cancer Research, Inc., March 30–April 1, 1942, Boston, Mass. (*1*).

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[4] Low-molecular-weight polypeptides such as glutathione and sulfhydryl or disulfide compounds such as cysteine or cystine do not give the characteristic catalytic wave in the absence of divalent cobalt or do so in the presence of trivalent cobalt only at concentrations much greater than the normal physiologic range. In any event, the low-molecular-weight sulfur compounds would be removed on dialysis of the sulfosalicylate filtrates. The finding (table 3) that there is little change in the height of the "filtrate wave" following dialysis indicates that little, if any, of the polarographic effect obtained can be attributed to them.

[5] Starting with early papers of Oriel and Barber (*13, 14*), a considerable body of literature has developed in regard to immunologic differentiation of urinary proteoses, especially proteoses obtained during allergic conditions. In spite of considerable early controversy, definite immunologic specificity now seems established for various proteoses. For example, Stull and Hampton (*15*) have recently shown that primary or secondary proteoses prepared by ammonium sulfate precipitation and dialysis of peptic digests of chicken, beef, beef fibrin, casein, and whey were antigenic and showed specificity as determined by the Dale test with guinea pigs.

studied. The proteose-filtrate-wave test ordinarily gives a far greater differentiation between normal and cancer bloods or serums than does the older protein wave test first employed by Brdicka, since the relative increases in proteose in cancer bloods or serums are much greater than the relative decreases in protein. Whole blood rather than serum has not to our knowledge been employed in polarographic studies before, except by Brdicka (*3*), who, however, first carried out a preliminary denaturation with alkali (cf. footnote 7). Blood gives considerably smaller waves than its serum, even after both are calculated to the same original blood volume. The smaller values with blood are probably owing to greater loss during filtration by adsorption on the additional hemoglobin protein precipitate present. It is also conceivable that serum proteins undergo more rapid or more extensive breakdown during the period of analysis. In this latter connection, the question remains whether some or even any of the proteose in blood or serum is present as such or as some precursor that rather readily breaks down during analysis to give the proteose measured. Furthermore, the exact degree of polarographic equivalence of different polarographically active materials of polypeptide nature, on either a weight, nitrogen, or cystine or other amino acid basis, calls for careful investigation.

POLAROGRAPHIC FILTRATE WAVES OF RAT BLOOD

The work reported in this section was carried out for the most part with the oxalated cardiac whole blood of Osborne-Mendel rats. Two-tenths of a cubic centimeter of blood was added to 1.8 cc. isotonic saline, and 2 cc. of 10 percent sulfosalicylic acid was added to the mixture. After 10 minutes the precipitated proteins were filtered off through Whatman No. 5 paper, and 1 cc. of the filtrate was added to 2 cc. of the hexammine cobaltic chloride reagent.[6]

[6] The reagent had the following constitution and was used within a few hours after its preparation: $Co(NH_3)_6Cl_3$, 0.001M; NH_4Cl, 0.1M; NH_4OH, 0.7M; KCl, 0.1M. One cubic centimeter of saturated caffein solution was added per 100 cc. of this reagent to suppress oxygen and cobalt maxima.

Polarograms were made on this solution at 21°-23° C. using a Leeds & Northrup Electrochemograph with the sensitivity adjusted so that the reagent control caused a galvanometer current deflection of 33 scale divisions at −1.5 volts (with respect to saturated calomel half cell). The significant height of the wave was taken to be the difference in deflection between the reagent control polarogram at −1.6 volts and the peaks of the experimental polarogram, which occurred at between −1.6 and −1.7 volts. All polarograms were thus strictly comparable in arbitrary deflection units. Under the precise experimental conditions just outlined [7] and over the lower part of the concentration curve, a deflection of one scale division corresponded roughly to 1 mg. proteose-nitrogen/100 cc. of blood. One tenth of a milligram of the isolated blood proteose described later, polarographed with 1 cc. of water and 2 cc. of the hexammine cobaltic chloride reagent, gave a polarographic wave of 10 scale divisions.

Typical polarographic filtrate waves for blood from a normal rat and from a rat with a large transplanted tumor (hepatoma 31, a liver tumor originally transplanted from a rat fed *p*-dimethylaminoazobenzene (*19*), are shown in figure 1.

Typical changes in polarographic waves following the implantation of tumors into rats are shown in figure 2. The numbers over the curves represent, in square millimeters, the product of the longest and shortest diameters of the tumors. The growth or regression of the tumors was paralleled approximately by corresponding changes in the polarographic filtrate waves. Extirpation of

[7] The procedure outlined must be followed explicitly if reproducible results are to be obtained. For instance, the filter paper used was 9 cm. in diameter. This apparently trivial detail is important inasmuch as the paper may absorb some proteose. Likewise, consideration of the given dilution is required since the protein precipitate absorbs some proteose, with the result that the wave is not always increased proportionally as the quantity of blood employed is increased; and furthermore our test differs from tests used hitherto by other workers in that no preliminary denaturation of the blood is called for prior to the sulfosalicylic acid treatment. Waldschmidt-Leitz and Mayer (*6*) used heat denaturation, and Brdicka (*3*) also used alkali denaturation and obtained higher filtrate waves than with undenatured human serums. We likewise have obtained higher waves with denatured human serums, but smaller waves with denatured rat or rabbit serums, no matter how time or strength of alkali was varied.

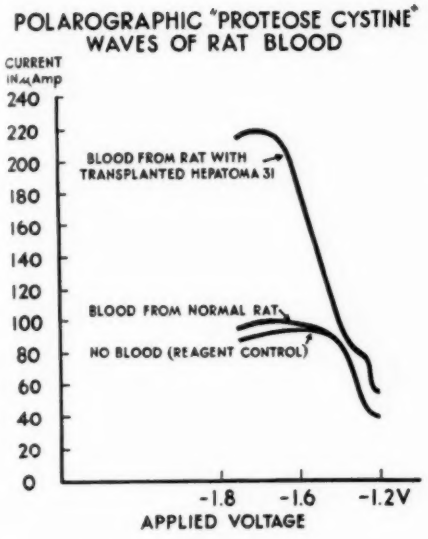

FIGURE 1.

well-established tumors was followed by a return of the filtrate wave to approximately normal values within a few days Large transplanted liver tumors irradiated with X-ray doses of 4,000 r ceased growing, but there was no decrease in blood proteose from the high level occurring before irradiation for at least 14 days, after which ulceration commenced.

A large number of experiments were carried out in order to determine the normal limits of the rat polarographic filtrate wave and the influence of various conditions upon this level. For comparative purposes, specimens of the bloods of other species were analyzed in exactly the same way. The results are briefly summarized in table 1.

The following conclusions have been drawn:

(1) The polarographic filtrate wave of normal rat blood was not significantly affected by strain, sex, age, fasting, biotin deficiency, sulfaguanidine feeding, or liver cirrhosis due to feeding of *p*-dimethylaminoazobenzene.

(2) The presence in rats of primary or transplanted tumors several square millimeters or greater in size led to significant increases in the polarographic filtrate waves.

(3) Acute and chronic infections such as pneumonia and chronic cecitis caused large increases in the polarographic filtrate waves of rat blood similar to those found in cancer.

(4) Production of severe tissue injury or shock was usually followed by polarographic filtrate waves definitely higher than normal.

(5) The presence of rapidly growing regenerating liver (3 days after partial hepatectomy) caused only a slight increase in polarographic filtrate waves, but no more than did operative controls. In pregnancy there was a small increase in the filtrate wave just before parturition.

(6) The subcutaneous or intraperitoneal injection of large amounts of homogenized healthy or necrotic tumor tissue or rat liver had only a small effect on the polarographic filtrate wave.

(7) The order of increasing polarographic filtrate waves of normal bloods of several species was apparently mouse, rabbit, rat and man, and guinea pig. Increases in wave heights in animals

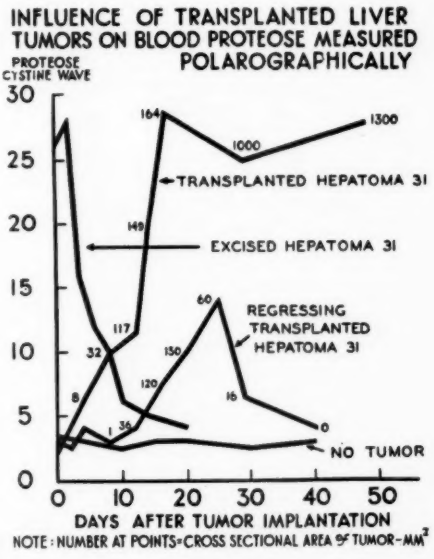

FIGURE 2.

TABLE 1.—*Polarographic filtrate waves of various normal and pathologic bloods and serums*

Source of blood	Individuals analyzed	Condition	Polarographic filtrate wave (scale divisions)	
			Extremes	Average
	Number			
Rats	248	Normal[1]	2– 5	3.0
		Cancer-bearing:		
Do	11	Jensen sarcoma (400–1,000 mm.[3])	13–33	24.3
Do	16	Primary hepatomas induced by feeding *p*-dimethylaminoazobenzene (20–1,000 mm.[3]).	8–44	20.5
Do	29	Hepatoma 31 (10–60 mm.[3])	3–14	7.5
Do	21	Hepatoma 31 (60–500 mm.[3])	8–28	18.8
Do	33	Hepatoma 31 (500 mm.[3])	19–40	29.0
		Infected:		
Do	23	Chronic cecitis	14–35	24.8
Do	7	Acute pneumonia	11–22	15.8
		On special diets:		
Do	8	Fasted 5 days	1– 3	2.5
Do	9	Biotin deficiency caused by egg-white feeding	1– 4	2.9
Do	10	Liver cirrhosis caused by *p*-dimethylaminoazobenzene feeding	2– 7	3.6
Do	9	Fed sulfaguanidine on synthetic diet 48 days	2– 3	2.7
		Pregnant:		
Do	5	11 days	3– 6	3.7
	7	20 days	4–10	5.9
		Injured:		
Do	5	Hind quarters scalded 20 seconds at 60° C. (ether anesthesia) — 3 hrs. after	3– 7	4.2
		Hind quarters scalded 20 seconds at 60° C. (ether anesthesia) — 18 hrs. after	5– 9	6.8
Do	2	Hind limbs crushed (ether anesthesia), — 3 hrs. after	4– 5	4.5
		Hind limbs crushed (ether anesthesia), — 18 hrs. after	5– 9	7.0
Do	6	5 gm. homogenized rat liver injected intraperitoneally — 3 hrs. after	3– 6	4.0
		5 gm. homogenized rat liver injected intraperitoneally — 18 hrs. after	3– 8	4.7
Do	5	1 gm. homogenized healthy or necrotic portions of hepatoma 31 injected intraperitoneally or subcutaneously. (24 hrs. after	3– 4	3.6
Do	5	Partial hepatectomy (nembutal anesthesia) — 18 hrs. after	4–10	7.0
		Partial hepatectomy (nembutal anesthesia) — 3 days after	3– 8	4.6
C3H mice	11	Normal	0– 1	.5
Rabbit	21	....do	5– 2	1.1
Do	3	Shope virus papilloma (2–3 weeks)	1	1.0
Do	10	Shope virus papilloma (10–30 weeks)	1– 3	2.0
Do	19	V2 carcinoma (10–20 days)	3– 8	4.4
Do	5	Brown-Pearce carcinoma (20 days)	2– 4	2.6
Guinea pig	4	Normal	5– 7	6.2
Dog (serum)	4	....do	5– 7	6.8
Man (serum)	125	....do	5–11	7.1
Do	17	Cancer of various types	10–14	12.4
Do	6	Lobar pneumonia	10–16	14.5
Do	8	Pulmonary tuberculosis	9–17	13.7

[1] Male and female albino rats weighing 40 to 500 gm. and of three different strains (Osborne-Mendel, Buffalo, or Cornell) were used with no significant differences.

[2] A mouse tested, bearing a spontaneous mammary tumor of 300 mm.[3] gave a wave of 2.0, or four times the normal average.

with cancer have been found regularly in rabbit, rat, and man.

(8) Early Shope virus papillomas a few weeks old, with extensive growth, did not produce increased blood-filtrate waves in rabbits (nor did well-developed Shope virus fibromas a week or 2 old), but 10–to–30–week old papillomas (that may have borne some infection) produced recognizable (ca. twofold) increases weeks or months before the onset of malignancy, which did not occur until the thirtieth to fiftieth weeks or longer. Uninfected, malignant, 10–to–20–day–old transplantable V2 carcinomas, however, produced a fourfold average increase, so that a polarographic distinction could be made between the malignant, and benign state in this homologous series in the rabbit. Healthy Brown-Pearce carcinomas also gave polarographic waves well above normal.

QUANTITATIVE STUDIES ON PROTEOSE IN RAT BLOOD

Early workers *(20–29)* demonstrated the presence of polypeptides in trichloracetic acid filtrates of serum and urine. They found that the concentration of these polypeptides was increased in patients with cancer or with certain other diseases. These workers determined the nitrogen or tyrosine in trichloracetic and phosphotungstic acid

filtrates of serum. The difference in nitrogen or tyrosine content of the filtrates was called the "index de polypeptidémie," since phosphotungstic acid was known to precipitate all polypeptides whereas trichloracetic acid left some in solution. Trichloracetic acid filtrates of normal human serum contained about 7 mg. percent of polypeptide nitrogen and about 1.5 mg. percent polypeptide tyrosine, whereas patients with certain diseases, including cancer, had an index of polypeptidemia as high as 20 mg. percent polypeptide nitrogen and 5 mg. percent polypeptide tyrosine.

It appeared probable that the materials giving the polarographic filtrate wave and the index of polypeptidemia were the same. This surmise is supported by the data in table 2, which shows the nitrogen content of tungstic acid, trichloracetic acid, and sulfosalicylic acid filtrates of blood from normal rats, rats with chronic cecitis, and rats with large transplanted tumors, as well as the polarographic waves of the tungstic acid and sulfosalicylic acid filtrates. The polarograph cannot readily be used to determine proteose in trichloracetic acid filtrates since trichloracetic acid is itself reduced at the dropping mercury electrode and obscures the filtrate wave. It is evident from table 2 that the index of polypeptidemia and the polarographic filtrate wave agree closely in these experiments. We therefore conclude that essentially the same material is responsible for the increase in index of polypeptidemia and the increase in polarographic filtrate wave in the blood of rats with cancer or infections. Our analyses of human serums have shown that the relations are similar to those found in rat blood.

Another method of determining blood proteose is to assay the total and the nondialyzable nitrogen in sulfosalicylic acid filtrates. The nitrogen content of the undialyzed filtrate represents the sum of the dialyzable nonprotein nitrogen and the proteose nitrogen, while the dialyzed filtrate contains proteose nitrogen alone. Results of such an experiment on blood from normal rats and from rats with large transplanted tumors are given in table 3. It is evident that the values for proteose nitrogen agree with those shown in table 2 obtained from the difference in the nitrogen contents of tungstic acid and sulfosalicylic acid filtrates. The magnitude of the polarographic filtrate wave of sulfosalicylic acid filtrates was unaffected by dialysis. It is evident that there was little or no polarographically active material in the dialyzable nonprotein nitrogen or in the tungstic acid filtrates. Also, whereas there was a striking difference in proteose content between normal and cancer-bearing rat blood, the dialyzable nonprotein nitrogen was essentially the same.

TABLE 2.—*Nitrogen content and polarographic waves of 2 percent tungstic acid, 30 percent trichloracetic acid, and 5 percent sulfosalicylic acid filtrates of rat blood*

Sources	Filtrate nitrogen content [1] [2]			Filtrate index of polypeptidemia [1] [2]		Polarographic filtrate wave [2]	
	TA	SSA	TCA	SSA–TA	TCA–TA	TA	SSA
Normal rat	44.5	49	48	4.5	3.5	0	4
Do	48.5	51	49	3.5	1.5	0	3
Do	44	47.5	47	3.5	3	0	3
Small hepatoma 31 [3]	46.5	55.5	57	9	10.5	0	10
Large hepatoma 31 [4]	49	61	56	12	7	0	14
Do	45	60	55	15	10	0	20
Do	63	81	75	18	12	0	27
Large Jensen sarcoma [4]	47	62.5	61	15.5	14	0	19
Do	46	53.5	53.5	7.5	7.5	0	13
Chronic cecitis	47.5	60	57	12.5	9.5	0	11
Do	45	55.5	55.5	10.5	10.5	0	11
Do	44	55	53.5	11	9.5	0	9

[1] In milligrams per 100 cc. of blood.
[2] TA, tungstic acid; TCA, trichloracetic acid; SSA, sulfosalicylic acid.
[3] Nonulcerated tumors. Product of long and short diameters=100 mm.^{2}
[4] Nonulcerated tumors. Product of long and short diameters=500 mm.^{2}

TABLE 3.—*Nitrogen in dialyzed and nondialyzed sulfosalicylic acid filtrates of rat blood*

Source	Total sulfosalicylic acid soluble nitrogen[1] (before dialysis)	Proteose nitrogen[1] (nondialyzable nitrogen)	Dialyzable nitrogen[1] (by difference)	Polarographic filtrate wave	
				Before dialysis	After dialysis
Normal rat blood.....	42	4.5	37	4	3.5
Do.....	43	4	39	3.5	3
Do.....	38	3.5	34	3.5	3
Do.....	47	3.5	43	3	2
Do.....	42	4.5	37	3	3
Do.....	46	3	43	2	2
Jensen sarcoma[2]....	83	46	37	41	43
Hepatoma 31[2]	78	41	37	37	37
Do.....	79	42	37	37	37
Do.....	66	28	38	29	29
Do.....	58	17	41	22	23
Do.....	53	17	36	22	20

[1] In milligrams per 100 cc. blood.
[2] Nonulcerated tumor. Product of long and short diameters=500 mm.^{2}.

After dialysis of the sulfosalicylic acid and trichloracetic acid filtrates for 24 hours, it was found that the trichloracetic acid filtrate gave a polarographic wave only slightly lower than the sulfosalicylic acid filtrate. This is in agreement with the findings on the nitrogen content of the filtrates and indicates that trichloracetic acid precipitates somewhat more nitrogen from blood than does sulfosalicylic acid.

ISOLATION OF PROTEOSE MATERIAL FROM RAT BLOOD

The material responsible for the polarographic filtrate wave has many of the properties of a protein. As Brdicka (*3*) has indicated, it is nondialyzable, contains cystine and tyrosine, is precipitated by tungstic and metaphosphoric acids, by colloidal Fe_2O_3, and by ethanol, and, as we have found, by about 70 percent saturated ammonium sulfate. It is, however, not coagulated by heat and not precipitated by sulfosalicyclic acid or by (20–30 percent) trichloracetic acid, whereas typical proteins are denatured and precipitated by these treatments. The material has, therefore, been referred to as proteose. Polarographically indistinguishable material can be produced by the action of alkali, acid, heating, or proteolytic enzymes on serum proteins, and can be further hydrolyzed by these agents.

The proteose material in the blood of normal rats or of rats bearing hepatoma 31 has been isolated (probably as a sulfosalicylic acid conjugate) by two different procedures. Fifty to one hundred cubic centimeters of pooled rat blood was mixed with an equal volume of water and 2 volumes of 10 percent sulfosalicylic acid. The precipitated proteins were filtered off after 10 minutes, and the filtrate was dialyzed 48 hours against tap water. The nondialyzable residue was then dried from the frozen state at low pressure. An alternative method of isolating the material involved the precipitation of the proteose from the sulfosalicylic acid filtrate with 6 volumes of ethanol and drying the residue in vacuo. Little or no polarographically active material was lost by either of these methods, as judged by polarographic measurements. Each of the methods yielded 30 to 60 mg. of dry solid per 100 cc. of pooled normal rat blood, and 90 to 200 mg. per 100 cc. of pooled blood samples from rats with tumors (large, transplanted hepatoma 31) and with average filtrate waves 3 to 6 times normal. Waldschmidt-Leitz (*30*) has mentioned briefly the preparation of similar residues he obtained by dialyzing sulfosalicylic acid filtrates of human serums and fractionating these with alcohol, but no analytical data were given. He stated that the polarographically active material in normal serum was a sulfur-containing protein split-product, while some of the polarographically active material in cancer serum was mucoid and sulfur-free. This latter observation has as yet not been confirmed.

The normal rat-blood proteose preparation obtained by dialysis against tap water showed somewhat lower nitrogen, sulfur, phosphorus, and carbohydrate, and a considerably higher ash content than that obtained with alcoholic precipitation. On calculating the former to an ash-free basis the two analyses became nearly the same (except for some difference in phosphorus content). The

TABLE 4.—*Chemical analyses of proteose materials prepared from sulfosalicylic acid filtrates of normal rat blood*

Component	Method[1]	Material prepared by dialysis	Material prepared by alcohol precipitation	
			Percent	Sulfosalicylic acid free basis
		Percent		*Percent*
Nitrogen	Digestion-nesslerization	6.4	9.2	15.9
Phosphorus	Fiske–Subbarow (*31*)	.05	.86	1.48
Sulfur	Parr bomb-turbidimetric	4.3	5.8	.86
Ash	Ignition in air	31.1	2.9	
Carbohydrate	Orcinol (*32*)	6.5	9.0	15.5
Extractable lipoid	Soxhlet ether extraction	(2)	(2)	(2)
Cholesterol	Acetic anhydride in H_2SO_4	(2)	(2)	(2)
Cystine	Hydrolysis-polarographic (*33*)	.5	.6	1.0
Methionine	McCarthy–Sullivan (*34*)	1.2	1.5	2.6
Amino nitrogen	Modified Van Slyke nitrous acid method		6.3	10.9
Protein equivalent	Biuret test—casein standard (*35*)	39	57	98
Sulfosalicylic acid	Calculated from total sulfur less cystine and methionine sulfur	31	42	0

[1] Italic numbers in parentheses refer to authors cited.
[2] Negative.

high ash content of the preparation dialyzed against tap water is presumably due to retention of bases.

The low nitrogen content of the isolated samples (6 to 9 percent) is not found in ordinary unconjugated proteins since the constituent amino acids have average nitrogen contents higher than this. The low nitrogen content is probably caused in part, at least, by the combination of the proteose with sulfosalicylic acid to form a soluble conjugate. This interpretation is based on the very high sulfur content of both the alcohol-precipitated and the dialyzed samples, a content much higher than could be accounted for as cystine and methionine sulfur. On the assumption that the extra (noncystine and nonmethionine) sulfur is present as the sulfur in sulfosalicylic acid combined with the proteose, the nitrogen values can be calculated to a sulfosalicylate-free basis. This calculation was readily made for the alcohol-precipitated proteose material (where the ash content is low), and as seen in the last colum of table 4, brings the nitrogen content to about 16 percent, a normal value for most proteins, and in agreement with the protein-equivalent content of the material as determined by a quantitative biuret test (*33*). The color given in the biuret test by this material is a violet indistinguishable from that given by casein, egg albumin, or the serum proteins, rather than the pink given by peptones, again suggesting that this material from blood has a relatively high molecular weight and should be classed as a proteose rather than as a peptone or lower polypeptide.

The foregoing preparative and chemical studies support the contention of Brdicka that the material in sulfosalicylic acid blood filtrates that is responsible for the polarographic filtrate wave is a protein split-product soluble in sulfosalicylic acid. In order to characterize the original proteose more thoroughly, it will be desirable to prepare the material by using methods not involving sulfosalicylic acid precipitation.

Possible similarities between the proteoses just described and other proteinaceous substances found in blood or urine in certain pathologic conditions have already been indicated briefly. In addition, there may be mentioned the presence of a proteinlike material soluble in trichloracetic acid recently found in the urine and probably plasma of certain patients with albuminuria (*36*). A similar "protein metabolite" has lately been reported in the urine and plasma of patients with severe burns (*37*). It is likely that these materials are similar to the proteoses reported here, although possibly of somewhat lower molecular weight since some dialyzed slowly through cellophane.

POLYSACCHARIDE C-REACTIVE PROTEIN

Another protein material resembling proteoses described in this paper has been observed in pathologic serums (*38, 39, 40, 41*) of patients with certain acute infections. This protein reacts with the nontype-specific C polysaccharide of pneumococcus to form a precipitate. We have found that treatment of blood from normal or tumor-bearing rats with polysaccharide C[8] has no influence on the polarographic filtrate waves obtained. Polysaccharide C forms no precipitate with these serums. Several serums from patients with acute lobar pneumonia were treated with polysaccharide C, and the precipitate formed was centrifuged down. Upon performing the usual polarographic filtrate tests on the supernatant solutions, we again found no decrease in the polarographic filtrate waves of these serums, as compared with those not treated with polysaccharide C. It seems likely, therefore, that the polysaccharide C reactive protein is not any appreciable part of the material responsible for the very high polarographic filtrate waves obtained with sulfosalicylic acid-treated serums from patients with lobar pneumonia. This is not surprising since, as we have found, the polysaccharide C reactive protein is precipitated by sulfosalicylic acid (or trichloracetic acid) and is thus a true protein as distinguished from a proteose.

After washing the polysaccharide C protein precipitate several times, dissolving it in alkali, and making the usual polarographic analysis, we found that it gave a polarographic wave that, on a nitrogen basis, was approximately as high as that obtained with the isolated sulfosalicylate-proteose, and therefore contained a relatively high content of unmasked cystine. It is evident that the polarograph might well be employed in analysis of polysaccharide C reaction protein as well as the proteose, under appropriate conditions.

[8] Obtained through the courtesy of Dr. O. T. Avery and Dr. W. T. Goebel, of the Rockefeller Institute for Medical Research, following suggestion of the present studies regarding it by Dr. Rollin D. Hotchkiss.

DISCUSSION

PROTEOSE SOURCE

There are four main theories as to the source of proteoses in blood. Fiessinger (*28*) and Cristol (*29*) believed that hyperpolypeptidemia might result (1) from a failure of the liver to metabolize the normal amounts of polypeptides formed, (2) from a failure of the kidney to excrete them normally, or (3) to an increase in their formation in certain tissues. Cancer, trauma, and many infections were thought to cause hyperpolypeptidemia through excessive protein decomposition in the affected tissues.

Brdicka (*3, 4, 5*), on the other hand, suggested that the material giving the polarographic filtrate test comes from the degradation of serum proteins, possibly through the action of the protective enzyme of Abderhalden. That proteoses similar to those described in this paper can arise from the serum proteins is evidenced by the fact that normal or cancer serum proteins allowed to remain at room temperature for as litle as 24 hours invariably show higher polarographic filtrate waves, often twice as high. Crossley, Kienle, Vassel, and Christopher (*18*) concluded from kinetic studies that the serum proteins of dogs infected with pneumococcus were partially degraded in vivo to molecules of lower weight. Brdicka (*4*) also showed that the parenteral injection of relatively small amounts of decocted milk into a normal individual led in a few hours to fever and a twofold increase in the polarographic filtrate wave. The amount of protein in the milk injected was insufficient to account for the increase, and the conclusion was reached that the foreign protein reaction had led to the breakdown of serum proteins possibly through action of a protective enzyme.

Obviously, any determination of proteose source requires a consideration of quantitative aspects. Data in this paper show that the amount of proteose in normal rat blood represents only a very small fraction of the total protein or polypeptide material in normal serum. The value of 4 mg. proteose nitrogen per 100 cc. of blood (tables 2 and 3),

multiplied by the usual protein factor of 6.25, gives 25 mg. percent proteose in normal rat blood. This is in agreement with the amount isolated from normal rat blood (30 to 60 mg. percent of the sulfosalicylic acid derivative) and represents only about 1/300 of the total serum protein. The value of 25 mg. percent is minimal, since it is very likely that some of the material was adsorbed and lost on the original protein precipitate incidental to the filtration during isolation.

The proteose in the blood of tumor-bearing rats may attain a concentration of 300 mg. percent, or 50 mg. percent as proteose nitrogen. This is roughly 10 times the amino nitrogen, about the same as the nonprotein nitrogen, one-sixth the globulin nitrogen, one-twentieth the albumin nitrogen, and one-thirtieth the total nitrogen in normal blood. It is also approximately equal to the total nitrogen in the cancers most actively capable, per unit of tissue, of causing its appearance in the blood. These considerations would seem to preclude tumor nitrogen, blood globulin nitrogen (with its low cystine and low polarographic activity), blood nonprotein nitrogen or blood amino nitrogen as being the main ultimate source of high blood proteose in tumor-bearing animals. On the other hand, whenever measured (either chemically or polarographically), the decrease in serum albumin has always been definitely greater than the accompanying increase in blood proteose and is logically indicated as the chief quantitative source of the latter, as already suggested qualitatively by Brdicka. In regard to tissue nitrogen as a source, it may be noted that liver cirrhosis and various injuries (table 1) were incapable of producing high-blood-proteose levels and also that the degree of necrosis in tumors was not an important factor.

The foregoing observations add likelihood to the view that the proteoses in blood of tumor-bearing animals originate largely, even if not wholly, from blood protein (albumin) rather than from the tumor nitrogen itself. Preliminary evidence of a kinetic nature may also be mentioned that is in harmony with this view, and that in addition indicates the tumor as a site of formation, even if not major chemical substrate source, of the blood proteose. Blood taken from large veins of well-developed tumors (hepatoma 31) was found to give 20 to 40 and in 1 instance 100 percent higher filtrate waves than cardiac blood taken from the same respective animals in which the filtrate waves were already 3 to 8 times normal; thus, where tumor-vein-blood filtrate waves were 30, 14, and 24, the respective cardiac-blood waves were 25, 10, and 12 (and normal-blood wave, 3). The localized nature of a tumor here makes for much more ready specification of the actual site of blood proteose formation, than in the case of many other pathologic conditions that produce proteose.

These considerations of ultimate chemical source and tissue site of proteose formation are not intended to implicate specifically chemical mechanism. Thus, blood protein (albumin) might be broken down entirely to simple peptones and amino acids and then rapidly rebuilt into proteoses, rather than taking the direct path of simple cleavage to proteoses. It may be that the tumor, in an effort to make its own protein, is responsible for this indirect path, and that the proteoses represent incidental or unused products of resynthesis from smaller molecules; or it may be that the proteoses are indeed formed by simple cleavage and represent a potential head of protein building blocks. However much or little may be known about the chemical source and tissue site of proteose formation, nothing definite is known as to its mechanism of formation.

PROTEOSE TOXICITY

Duval, Roux, and Coiffon (*27*), Fiessinger (*28*), and Cristol (*29*) have considered the possibility that the protein split-product in the blood of patients with cancer and other diseases are toxic to the patients and have suggested that surgical shock, cancer cachexia, and other systemic effects might result from the presence of these toxic proteoses. It is well known that severe systemic effects and shock may result from the re-

lease of the ligature from a ligated limb or from the injection of peptones or homogenized tissue into rats. The possibility exists that these effects may be due to the liberation of toxic proteoses into the blood stream. This possibility finds some support in table 1, which shows that the blood of severely injured rats contains some increased amount of proteose. Whether the proteose is toxic or is merely an incidental result of tissue injury is not yet known.

We have observed that 0.2 mg. per cubic centimeter of the proteose conjugates prepared from the blood of rats completely stopped the growth of yeast, *Saccharomyces cerevisiae,* Fleischman strain 139 (sulfosalicylic acid controls negative). Similar toxicity was noted with 0.2 mg. per cubic centimeter of salmon protamine, and with 0.05 mg. per cubic centimeter of purothionine, a crystalline protein (or proteose) from wheat (Stuart and Harris (*42*)). Whether serum proteose is toxic in its native state to animal tissues is open to conjecture and merits study. It is suggestive, however, that the systemic effects of implantation and excision of tumors on the catalase activity of the rat liver (Greenstein (*35*, *36*)) and on the level of the blood proteose are remarkably parallel. It is possible that toxic proteoses bring about the reduction of liver catalase activity in animals bearing tumors.

SUMMARY

The present paper extends to experimental animals of various species the findings of Brdicka and earlier workers that serums of human cancer patients contain abnormally high amounts of protein split-products that possess the properties of proteoses and that may be determined either polarographically or chemically (by index of polypeptidemia, etc.).

Rat blood was found to contain a nondialyzable, heat-stable, cystine-containing proteose material that was precipitable by 70 percent saturated ammonium sulfate, alcohol, or tungstic acid, but not by sulfosalicylic acid or by 20 to 30 per cent trichloracetic acid. Sulfosalicylic acid filtrates of blood of normal rat blood contained 3 to 4 mg. proteose nitrogen per 100 cc. blood, whereas similar filtrates of blood of tumor-bearing rats attained levels of 20 to 50 mg. The dialyzable nonprotein nitrogen blood levels were essentially the same in normal and cancer-bearing rats, however.

Conditions that also led to increased blood proteose in the rat were pyogenic infections (pneumonia, cecitis) and to a lesser extent severe injuries and advanced pregnancy. Without notable effect were strain, sex, age, diet, liver cirrhosis, and regenerative growth of liver. Regression or extirpation of tumors was accompanied by rapid return to normal blood proteose levels. In rabbits, transplanted malignant tumors (V2 and Brown-Pearce carcinomas) gave blood proteose values several times normal, but young homologous benign tumors (Shope virus papillomas) did not.

Proteose preparations were obtained from the blood of normal rats and of rats bearing hepatoma 31 by precipitating the serum proteins with sulfosalicylic acid and separating out the residual proteose either after dialysis and drying from the frozen state or by alcohol precipitation. Analyses on such materials are reported for amino nitrogen, cystine, methionine, carbohydrate, lipid, phosphorus, sulfur, and ash. The isolated material was found to contain a very large amount of sulfur not accounted for by cystine or methionine, indicating that the proteose was combined with sulfosalicylic acid.

Polysaccharide C-precipitable protein, which occurs in certain pathologic serums, was found to give a polarographic filtrate wave of the same order as that given by serum proteoses. Polysaccharide C formed no precipitate with various cancer, pathologic, or normal serums tested and did not affect the polarographic filtrate waves given by such serums; nor did it affect the filtrate wave given by pneumonia serums actually containing the polysaccharide C-precipitable protein, since the latter is eliminated by sulfosalicylic acid precipitation prior to polarographic wave measurement.

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